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GD2-targeted immunotherapy and radioimmunotherapy

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Abstract

Ganglioside GD2 is a tumor-associated surface antigen found in a broad spectrum of human cancers and stem cells. They include pediatric embryonal tumors (neuroblastoma, retinoblastoma, brain tumors, osteosarcoma, Ewing's sarcoma, rhabdomyosarcoma), as well as adult cancers (small cell lung cancer, melanoma, soft tissue sarcomas). Because of its restricted normal tissue distribution, GD2 has been proven safe for antibody targeting. Anti-GD2 antibody is now incorporated into the standard of care for the treatment of high risk metastatic neuroblastoma. Building on this experience, novel combinations of antibody, cytokines, cells and genetically engineered products all directed at GD2 are rapidly moving into the clinic. In the review, past and present immunotherapy trials directed at GD2 will be summarized, highlighting the lessons learned and the future directions.

Keywords

GD2; neuroblastoma; immunotherapy; cytokines; cytotherapy

Introduction

Targeting tumor associated antigens (TAA) with immune effectors, including monoclonal antibodies (MAbs) and T-cells, has achieved substantial clinical benefits in cancer therapy, thereby energizing scientists and the pharmaceutical industry in the past decade. The goal is to find a target with ideal efficacy and safety profile, while continuing to optimize immune effectors. The context where TAA targeted therapy is applied can be critical for its success. For example, the leading cause of cancer treatment failure is minimal residual disease (MRD), i.e. the small number of cells that survive conventional therapy which in turn provide the nidus for regrowth or new metastasis. Immunotherapy targeting TAA holds promise for the eradication of MRD by virtue of its tumor-selectivity with toxicities that do

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not overlap with those of conventional chemotherapy or radiation therapy. Here, we review GD2-directed therapy, with an emphasis on immunotherapy and radioimmunotherapy applied to neuroblastoma and other GD2-positive tumors.

GD2, a disialoganglioside, is an oncofetal antigen that is expressed in the fetus. It is also found on neural stem cells,[1] mesenchymal stem cells,[2] and breast cancer stem cells.[3,4] Postnatally it is detected on peripheral neurons, central nervous system and skin melanocytes.[5] Its biological role is poorly understood. It is probably important in mediating attachment of tumor cells to extracellular matrix proteins.[6] Since the first report on GD2 as a TAA in neuroblastoma,[7] much is still unknown about its function in developmental biology or in oncogenesis. Compared to other antigens, GD2 expression is high and relatively homogeneous between and within neuroblastoma tumors, although heterogeneity has also been described.[8] Based on multiple criteria (e.g. therapeutic function, immunogenicity, role of the antigen in oncogenicity, specificity, expression level and percent of antigen-positive cells, stem cell expression, number of patients with antigen-positive cancers, number of antigenic epitopes, and cellular location of antigen expression), the National Cancer Institute program for prioritization of cancer antigens ranked GD2 12th among a list of top 75 cancer antigens.[9] Its rank becomes even higher if only directly targetable antigens are selected. In addition to neuroblastoma, other malignant tumors such as melanoma,[10] soft tissue sarcomas,[11] osteosarcoma,[12] desmoplastic small round cell tumor (DSRCT),[13] small cell lung cancer (SCLC)[14] express GD2, although with more heterogeneity by immunohistochemistry.

Neuroblastoma is the most common extracranial solid tumor of childhood. About 90% of patients are diagnosed before their 6th birthday, with a median age of 19 months. In contrast to low and intermediate risk neuroblastoma (50% of cases), long term prognosis for high-risk neuroblastoma remains dismal.[15] The introduction of murine monoclonal antibodies (e.g. 3F8[16] and 14G2a[17]) specific for the penta-saccharide moiety of GD2 initiated a new era of neuroblastoma immunotherapy more than 2 decades ago. The landmark randomized trial carried out by the Children's Oncology Group (COG) definitively demonstrated an overall survival benefit of anti-GD2 antibody ch14.18 when combined with cytokines (interleukin-2 (IL-2) and granulocyte–macrophage colony stimulating factor (GM-CSF)), and anti-GD2 antibody therapy has now become the standard of care for patients with high-risk neuroblastoma.[18] Humanized 3F8 (hu3F8)[19] is now proven safe and non-immunogenic in phase I clinical trials.[20,21] Granulocyte-mediated antibody dependent cell-mediated cytotoxicity (ADCC)[22] and natural killer (NK) cell mediated NK-ADCC[23] are important effector mechanisms. Despite the small number of patients for this orphan disease (650 new cases in the US each year and only 50% with high risk disease), important lessons in antibody immunotherapy have been learned.

Anti-GD2 monoclonal antibodies

Murine antibodies: IgG3 antibody 3F8 (see table 1 for clinical trials)

In the first-in-human phase I study of anti-GD2 MAb, toxicity of murine IgG3 antibody 3F8 was assessed in 17 patients with neuroblastoma and malignant melanoma.[24] Tumor response by using International Neuroblastoma Response Criteria (INRC)[25] was observed in 2 of 17

patients with neuroblastoma.[26] Importantly, this study demonstrated specific binding of 3F8 antibodies to tumor using in vitro immunostaining and in vivo radioimaging with ^{131}I. Patients with significant levels of circulating human-anti-mouse antibody (HAMA) had minimal side effects, but also no therapeutic benefits from immunotherapy. Those with level of HAMA <1,000 U/mL had many side effects together with therapeutic responses. Toxicity profile included hypertension, fever, urticaria and pain. In subsequent studies it was shown that reduction of pain side effects could be achieved with pretreatment with heat-modified 3F8 to desensitize peripheral nerves. This approach was tested in Phase I clinical trial, showing feasibility of 3F8 dose-escalation without increase in analgesic requirements.[27] In all subsequent trials 3F8, was only given if the HAMA test was <1,000 U/mL.

Clinical benefit was demonstrated in a phase II trial where 3F8 alone was used for remission consolidation in patients >1 year of age with stage 4 neuroblastoma.[28] At study entry most of the patients had complete or very good partial remission (CR/VGPR), albeit 74% of these patients were found to have MRD when tested by non-conventional methods including immunoscintigraphy, immunocytology, quantitative (q)RT-PCR. This study demonstrated that long-term remission (>20 years) could be achieved with 3F8 treatment without myeloablative therapy (e.g. autologous marrow or stem cell transplantation).

In subsequent studies 3F8 was combined with cytokines, β-glucans, or cellular immune effectors. A phase I study of 3F8 combined with escalating doses of IL-2 was terminated after 12 patients because of capillary leak syndrome and unfavorable pharmacokinetics (unpublished results). Addition of intravenous (iv) GM-CSF led to enhancement of granulocyte-mediated ADCC.[29] In a Phase I trial in patients with refractory chemo-resistant neuroblastoma in the bone marrow (BM), response was achieved in 12/19 patients with primary refractory, and 5/10 with secondary refractory neuroblastoma.[30] It was shown that the use of qRT-PCR for detection of specific markers of MRD served as surrogate to achieve more precise evaluation of response to 3F8 therapy and patient stratification.[31] Subsequent studies on the polymorphism of *FCGR2A* gene which encodes the Fcγ-receptor IIA (CD32), demonstrated a correlation between the high affinity allele and clinical outcome in patients treated with 3F8 and GM-CSF. These findings imply that genetic polymorphism can predict therapeutic efficacy and might be a potential tool for selection of patients who can most benefit from therapy.[32] In a subsequent trial,[33] 79 patients with persistent osteomedullary neuroblastoma documented by histology and/or metaiodobenzyl-guanidine (MIBG) scan were treated with 3F8 plus subcutaneous (sc) GM-CSF, while isotretinoin was also included after remission was achieved. Complete response rates to 3F8+scGM-CSF were 87% by histology and 38% by MIBG. Five-year progression-free survival (PFS) was 24%±6%, which was significantly superior to 11%±7% with 3F8+ivGM-CSF (p=0.002); five-year overall survival (OS) was 65%±6%. In a multivariate analysis, significantly better PFS was associated with R/R or H/R *FCGR2A* polymorphism, sc route of GM-CSF, and early MRD response. *MYCN* amplification was not prognostic of outcome. Complement consumption was similar with either route of GM-CSF. Toxicities were manageable, allowing outpatient treatment.

A summary of 169 patients with high risk neuroblastoma, i.e. stage 4 diagnosed at ≥18 months of age or those with *MYCN* amplification treated in their first remission with 3F8 ±

GM-CSF ± isotretinoin, showed that at 5 years from the start of immunotherapy, PFS improved from 44% (3F8 only) to 56% (3F8+ivGM-CSF) and 62% (3F8+scGM-CSF). OS was 49%, 61%, and 81%, respectively. Relapse was mostly at isolated sites (CNS, soft tissues and marrow/bone). Independent adverse prognostic factors included difficulty getting into first remission and positivity of marrow MRD by qRT-PCR after 2 cycles of immunotherapy (post-MRD), whereas favorable prognostic factors included missing ligand for inhibitory killer immunoglobulin-like receptor (KIR), positive HAMA response, and scGM-CSF.[34] Phase II studies of high dose 3F8 (4-fold higher) in combination with GM-CSF (NCT01183429, NCT01183884, NCT01183897) currently recruit patients and the outcome analyses will be done.

The potential of 3F8 to control MRD was further tested in 101 patients with ultra high risk neuroblastoma, i.e. stage 4 neuroblastoma diagnosed after 18 months of age or with *MYCN* amplification, whose disease progressed before being salvaged back into ≥2nd remissions.[35] They were treated with 3F8/GM-CSF plus isotretinoin. PFS at 48 months was 33%±5%. Time from 3F8 to progression was longer than the time to first relapse by >3–6.5 fold (n=17 patients), >1.5–1.9 fold (n=10), and >1–1.49 fold (n=10). Among the 33 patients who continued progression-free with long follow-up (>28–111 months), 18 had *MYCN*-amplified NB, 16 without maintenance chemotherapy and only two underwent autologous stem-cell transplantation (ASCT) during salvage. Patients sensitized to 3F8 were treated with rituximab to reduce HAMA to <1,000 U/mL before 3F8 treatment. By multivariate analysis, age, post-MRD response and maintenance chemotherapy were significant favorable prognostic factors for PFS. Variables significant for overall survival were post-MRD response and rituximab.

β-glucan, a natural polymer of glucose, interacts with CR3 and CR4 on myeloid cells, enhancing adhesion to their natural ligands including iC3b, the breakdown product of complement activation. Through activation of these receptors, anti-tumor effect of monoclonal antibodies can be enhanced in xenograft models.[36] A phase I study of oral barley-derived β-glucan (10 to 80 mg/kg/day for 10 days) when combined with iv 3F8 (10 mg/m^2/day × 10 days) demonstrated acceptable safety profile without reaching glucan maximum tolerated dose (MTD). Improvement was observed in 13 in 22 patients by MIBG scintigraphy.[37] A subsequent phase I study using 10 to 200 mg/kg/day × 17 days of oral yeast-derived β-glucan (NCT00492167) did not reach glucan MTD, while the study did show improvement in MIBG scans in 7/24 and CR of BM in 3/5, with an overall response of 1 complete response (CR), 1 partial response (PR), 22 stable disease (SD), and 17 progressive disease (PD).[38] The phase II combination study with 3F8, GM-CSF, β-glucan and isotretinoin (NCT00089258, completed) was discontinued because of a high incidence of HAMA (unpublished data).

A pilot study combining 3F8 with allogeneic NK cells (NCT00877110) used haploidentical NK-cells optimized for anti-neuroblastoma cytotoxicity by selecting NK donors: from licensed NK-cells responding to “missing self (MS)” or from unlicensed NK-cells responding to “missing ligand (ML)” (see Lessons Learned).[39] Patients received a lymphodepleting regimen of high-dose cyclophosphamide, topotecan and vincristine (days 1–3) prior to infusion (day 5) of NK-cells isolated from donor leukophereses using CD3-

depletion (to $<2\times10^4$ CD3+ cells/kg) followed by CD56-enrichment. For subsequent cycles (≤3), conditioning was reduced to cyclophosphamide alone. 3F8 (20 mg/m^2/day) was administered on days 8–12. 20 patients received 23 cycles: 1, 9, 5 and 5 at dose-level 0 ($<1\times10^6$ CD56(+) cells/kg), I ($1–4.99\times10^6$), II ($5–9.99\times10^6$) and III ($10–30\times10^6$), respectively. 7, 7 and 6 donors had ML, ML+MS and neither, respectively. MTD has not yet been reached. One patient had dose limiting toxicity (DLT): grade 4 hypertension and vomiting. The only other >grade 2 possibly-related toxicity was transient hepatic transaminitis. Neither graft versus host disease (GvHD) nor myeloablation was observed. Responses by INRC (n=19) were: 3 complete remission (n=3), 13 stable disease (7/13 had improved MIBG scores [median-4]), and 3 progressive disease (all donors without ML or MS).

Murine antibodies: IgG2a antibodies 14.G2a and ME36.1(see table 1 for clinical trials)

14.G2a is a class switch variant of 14.18 – IgG3 isotype of anti-GD2 antibody. Its phase I clinical trial in 18 patients demonstrated very modest antitumor activity[40] at the expense of significant toxicities. A subsequent COG study tested anti-tumor activity of 14.G2a in combination with IL-2.[41] Thirty-three pediatric patients with GD2(+) malignancies were included in the study. Partial response was observed in neuroblastoma (n=1/31) and CR in osteosarcoma (n=1/2). ME36.1 antibody is a mouse IgG3, class switched to IgG1 and IgG2a ME36.1. Unlike the other two anti-GD2 MAb 3F8 and 14.G2a, it cross-reacts with GD3.[42] Preclinical studies demonstrated ani-tumor activity,[43] but no clinical trials have been attempted.

Chimeric antibody ch14.8 (see table 2 for clinical trials)

One limitation of murine MAb is their interference by HAMA. Chimeric antibody retains murine variable heavy (VH) and variable light (VL) chains grafted onto human IgG constant domains. In the case of humanized antibodies, only murine complementarity determining region (CDR) domains are retained, while most of the framework VH and VL sequences are of human origin. Chimeric 14.18 (ch14.18) antibodies demonstrated significantly higher ADCC when compared to its murine variant in experiments in vitro.[44] Initial study in 13 patients with malignant melanoma did not show any anti-tumor response.[45] Human anti-chimeric antibody (HACA) was detected. Further trials in 9 patients with neuroblastoma showed promising results: CR (n=2), PR (n=2), and MR (n=1).[46] In another small study of 10 patients with neuroblastoma and 1 adult with osteosarcoma, one PR and 4 MR were seen. Seven patients developed HACA response.[47] Interestingly, all patients who developed high titers of HACA had therapeutic benefits. Ch14.18 was further assessed in larger trials in patients with newly diagnosed stage 4 neuroblastoma. In a retrospective study that included 334 evaluable patients, no clear therapeutic advantage of ch14.18 was elicited.[48] No statistically significant difference in event free survival (EFS) was observed in patients who received ch14.18 in comparison to those who were treated with oral low-dose maintenance therapy or did not receive any further treatment after initial therapy. An update of this study after 11 years of follow-up changed the original conclusion, observing a significantly better EFS and OS at 9 years for the immunotherapy group, although in the multivariate analysis, no difference was seen between immunotherapy and oral chemotherapy groups.[49]

Combination of ch14.18 with IL-2 and GM-CSF was subsequently evaluated in several clinical trials. In a COG phase I study, ch14.18 and GM-CSF were given to patients with neuroblastoma immediately after stem-cell transplantation. Nineteen patients were eligible for analysis. Disease progression occurred in 8 of 14 patients in first remission and 2 of 5 patients in second remission with a median follow-up of 40 months.[50] Phase II study with ch14.18 and GM-CSF demonstrated 1 CR, 3 PR and 1 MR in 27 patients with recurrent or refractory neuroblastoma.[51] Pharmacokinetics of ch14.18 when combined with GM-CSF is being studied in an ongoing trial (NCT01418495). Treatment with ch14.18 and rhGM-CSF in patients with metastatic melanoma demonstrated toxicity similar to that observed with ch14.18 alone without improvement in tumor response.[52] Combination with IL-2 was tested in a phase I trial in adult patients with melanoma. Antitumor activity included 1 CR, 1 PR, 8 SD. Induction of lymphokine-activated killer (LAK) cell activity was observed in this study.[53] In a phase III randomized study among patients with high risk neuroblastoma following autologous stem cell transplantation (NCT00026312), the combination of isotretinoin, ch14.18, IL-2 and GM-CSF was compared to standard maintenance therapy with isotretinoin alone. Randomization was stopped early because of the superiority of immunotherapy versus standard therapy with regard to EFS (66±5% vs. 46±5% at 2 years) and OS (86±4% vs. 75±5% at 2 years).[18] Ch14.18 antibody produced in Chinese hamster ovary (CHO) cells (rather than in mouse SP2/0 cells - see below) was tested in a phase I study in relapsed or refractory stage 4 neuroblastoma patients. Side effects and pharmacokinetics similar to those with ch14.18/SP2/0 were observed.[54] Another ongoing phase III randomized study of the European SIOP Neuroblastoma Group (SIOPEN) in high-risk neuroblastoma is testing the efficacy of isotretinoin and ch14.18/CHO, with or without IL-2 after autologous stem cell transplant (NCT01704716). Clinical response following long-term infusion of ch14.18/CHO in combination with sc IL-2 was assessed in high-risk neuroblastoma. The overall response following INRG guide lines was determined at 30% (12/40). The EFS was 32.4 % (observation 0.1 – 3.2 years, mean: 1.6 years) and an OS of 66.8% (observation 0.3 – 3.9 years, mean: 3.1 years).[55] Continuous infusion of ch14.18/CHO antibodies with sc IL-2 is currently under investigation in phase I/II study (NCT01701479). A combination of ch14.18 with lenalidomide (analogue of thalidomide) and isotretinoin is also being tested in a phase I design (NCT01711554). In a phase I trial of concurrent therapy with ch14.18 and R24 (anti-GD3) antibodies and IL-2 in patients with melanoma and sarcoma, MTD were lower than the MTD of each antibody in prior studies.[56]

Humanized antibodies hu14.18 and hu3F8

Humanized 14.18 (hu14.18) was tested in a phase I trial in children and adolescents with neuroblastoma, osteosarcoma and melanoma (NCT00743496). Dose-limiting grade 3 or 4 toxicities were observed in four of 36 evaluable patients with refractory and recurrent neuroblastoma and were characterized by cough, asthenia, sensory neuropathy, anorexia, serum sickness, and hypertensive encephalopathy.[57] Combination of hu14.18 with IL-2, GM-CSF and NK cells is the focus of a phase I study in children with relapsed or refractory neuroblastoma (NCT01576692). Efficacy of chemotherapy combined with hu14.18 plus GM-CSF is also being tested (NCT01857934).

3F8 was humanized (hu3F8) based on its crystal structure and manufactured in CHO cells.[8] In a phase I dose escalation study (NCT01419834)[20] hu3F8 (0.06 to 3 mg/kg/cycle) was given outpatient intravenously over 30 minutes, split as 2 and later amended to 3 doses per cycle; cycles were repeated every 3–6 weeks up to 24 months. Among 32 patients (neuroblastoma – 30, osteosarcoma – 2) two experienced a reversible but DLT of elevated liver transaminases; MTD has not been reached. At equivalent doses hu3F8 was less painful than murine 3F8. Human anti-human antibody (HAHA) was detectable in only 5 patients, despite their prior sensitization to murine 3F8 in 26 patients, and despite multiple cycles (up to 16) of hu3F8. Best responses on hu3F8 treatment were: CR (n=5) and PR (n=1), 21 (66%) maintained CR or SD (median 7 months, range 1–23+), and 5 (16%) developed PD.

In a second phase I dose escalation of hu3F8 in combination with GM-CSF (NCT01757626)[21] patients with relapsed high risk neuroblastoma received cycles of hu3F8/GM-CSF in the absence of HAHA up to 24 months. MAb (0.9–3.6 mg/kg/cycle) was infused iv outpatient over 30 min three times a week (i.e., 3 days/cycle). Daily GM-CSF was administered subcutaneously, beginning 5 days pre-hu3F8, through the last dose of hu3F8. Of the 18 patients enrolled to date, 9 were in the 2nd remission; nine had osteomedullary metastases, and 16 had prior anti-GD2 immunotherapy with murine 3F8 (n=13), ch14.18 MAb (n=3), or both (n=1). PK studies showed dose-dependent increases in peak serum concentration, but not in terminal half-life ($T_{1/2}$) (4.5±1.4 days). MTD has not been reached. HAHA developed in three patients (two previously treated with ch14.18).

In a third phase I dose escalation study of hu3F8 in combination with IL-2 (NCT01662804),[58] hu3F8 (starting dose at 0.3mg/kg/cycle, split as day 1 and day 8 infusions) was combined with a fixed dosage of scIL-2 (6×10^6 units/m^2/day x5 days/cycle starting on day 9), total of 4 cycles every 21 days, in patients with refractory or recurrent GD2(+) solid tumors. There was no DLT up to 0.9 mg/kg/cycle (n=8 patients). Side effects were grade 1/2 pain, allergic reactions, and fever. Pharmacokinetic studies showed dose-dependent increases in Cmax and Cmin. In 6/6 patients treated on the first two dosage levels (0.3 mg/kg and 0.6 mg/kg), significant discrepancies in hu3F8 $T_{1/2}$ were seen between doses 1 and 2 of a cycle: $T_{1/2}$ of dose 2 of hu3F8 (given with scIL-2) decreased by ~60% compared to $T_{1/2}$ of dose 1 (hu3F8 given without scIL-2). However, for 2/2 patients treated at dosage level 3 (0.9mg/kg), $T_{1/2}$ of dose 2 of hu3F8 was decreased by only ~5% compared to dose 1. Four out of 8 patients developed HAHA despite no prior anti-GD2 therapy. There have been no objective responses to date but 1 of 3 neuroblastoma and 2 of 4 osteosarcoma patients completed 3+ cycles without disease progression.

Non-compartmental pharmacokinetic analysis[59] of serum concentrations over time during cycle 1 of the phase I trial of hu3F8 (NCT01419834) revealed a dose-independent $T_{1/2}$ of 3.26±1.09 days. Both Cmax (R=0.95) and AUC (R=0.91) were strongly correlated with dose level, while Cmax was correlated with AUC (R=0.97). Pharmacokinetic parameters remained statistically unchanged during subsequent treatment cycles. When the PK of hu3F8 alone in the phase I trial (NCT01419834) was compared to that combined with IL-2 (NCT01662804),[58] at comparable dosage level, there was a strong positive linear correlation between patient's weight with AUC (R=0.95±0.11), Cmax (R=0.85±0.14), Cmin

(R=0.88±0.14), and negative correlation with Cl (R=0.93±0.42). No correlation was observed between $T_{1/2}$ and patient's weight.

GD2 vaccines

Even though MAb therapy is often regarded as passive immunotherapy, the induction of a host anti-tumor or anti-idiotype network following MAb therapy has raised the hypothesis that vaccination against GD2 may achieve more long term tumor control.[60] HAMA, an indirect measure of the host anti-idiotype network, was consistently correlated with long term survival.[34,60] Because of these observations, anti-idiotypic antibody vaccines such as mouse IgG1 antibody 1A7 specific for ch14.18[61] and rat IgG1 antibody A1G4 specific for 3F8[62] were tested in phase I trials (NCT00003023). The GD2 peptide mimotope[63] and its DNA vaccine[64] can induce serum antibodies and protective anti-GD2 IgG responses in mice. However, in contrast to whole-antigen vaccines, single-epitope vaccines will probably be limited by their narrow target coverage. The whole GD2 antigen has also been conjugated to keyhole limpet hemocyanin (KLH) to overcome the poor immunogenicity of carbohydrates.[65] In a phase I trial, the MTD of the immunological adjuvant OPT-821 (50, 75, 100 and 150 μg/m^2 per injection) was tested in combination with 30 μg each of GD2-KLH and of GD3-KLH, all administered subcutaneously (weeks 1–2–3–8–20–32–52) to 15 patients in ≥2nd remission (NCT00911560).[66] Oral yeast β-glucan (40 mg/kg/day, 14 days on/14 days off) started week 6 and continued through 12 months. At 150 μg/m^2 of OPT-821 (the dosing used in adults), no DLT was observed. 13/15 patients received the entire protocol treatment including 12 who remain relapse-free at 24+ to 39+ (median 32+) months and one who relapsed (single node) at 21 months. Relapse-free survival was 80±10% at 24 months. 12/15 patients had antibody responses against GD2 and/or GD3. Disappearance of MRD was documented in 6/10 patients assessable for response. The study was expanded to accrue more patients.

Immunocytokines

Fusion of cytokines to GD2-specific antibody has the potential to improve tumor delivery and help achieve a higher local concentration and reduce systemic toxicity. In phase I study of hu14.18/IL-2 in 28 children with refractory neuroblastoma and melanoma, no objective responses were observed. Hypersensitivity, hypotension and myelosuppression were dose-limiting toxicities.[67] Phase II study included 39 children with refractory neuroblastoma. Of 13 patients with measurable disease, 3 had SD. Among 23 patients with evaluable disease (based on BM histology and/or MIBG scintigraphy), 5 achieved marrow CR. Anti-hu14.18 antibodies developed in 40% of patients.[68] Toxicity profile was similar to that observed with IL-2 alone; they included capillary leak, hypoxia, pain, rash, allergic reaction, elevated transaminases, and hyperbilirubinemia. A Phase II study of hu14.18/IL-2 in combination with GM-CSF and isotretinoin in neuroblastoma (NCT01334515) and a phase II study among patients with minimal residual melanoma were recently completed (NCT00590824). Anti-GD2/GM-CSF immunocytokine showed enhanced ADCC in vitro when compared to antibody alone or in combination with systemic GM-CSF.[69] Despite these compelling data in syngeneic mouse models,[70] where anti-GD2 antibody targeted cytokines to tumor sites as immune stimulants when applied systemically[68] or locally,[71] iv hu14.18-IL-2 produced BM

remission in patients only if neuroblastoma tumor burden was minimal,[68] and no benefit was seen for soft-tissue tumors.[68,72] The clinical advantage of hu14.18-IL-2 over ch14.18 plus IL-2 is uncertain, given the grade 3 and 4 capillary leak with abnormal liver functions seen in both settings.[18,68] IL-15 is an alternative cytokine that activates NK and natural killer T-cells (NKT),[73] but without the severe side effects of IL-2.

Lessons learned from clinical trials of anti-GD2 antibodies

Importance of NK cells and the role of inhibitory killer cell immunoglobulin-like (KIR) receptor

Anti-GD2 MAb is more than a passive linker of tumors to NK cells; they rescue NK cells from suppression by neuroblastoma. FcγRIII (CD16)-mediated signaling removes the inhibition by KIRs.[74] Since 60% patients have KIRs on their NK cells with no corresponding KIR ligands during cellular maturation,[75] these NK cells are “uneducated” and hyporeactive until their CD16 is activated during ADCC. On the other hand, while “educated” NK cells are trained to kill, they are inhibited by tumor HLA upregulated during ADCC. This inhibition by HLA explains why patients with “missing inhibitory KIR ligands” tend to have longer survival following anti-GD2 MAb therapy.[23,34,75]

Importance of Myeloid ADCC

Granulocyte-ADCC of neuroblastoma is unique among human cancers.[34,69] This killing does not depend on oxidative intermediates, but requires azurophil (primary) granule exocytosis, and is enhanced by GM-CSF.[29,69] In ADCC, FcγRIIA (CD32) is the responsible receptor on granulocytes and myeloid effectors[76] and its high affinity allele (131-R, see Table 3) for MAb 3F8 is associated with a more favorable patient outcome.[32,75] Since complement receptors CR3 and CR4 on granulocytes are also important adhesion molecules for ADCC,[69,76] they can be manipulated to enhance tumor killing. One of their natural activating ligands is the membrane-bound C3bi, a breakdown product of C3 activation by MAb.[77] When CR3 is activated, it acquires the conformational neoepitope CBRM1/5,[78] which correlates with better patient survival.[22,34] β-glucan binds and activates CR3, and when administered orally, enhances antibody therapy of NB.[79] Tumor associated macrophages (TAM) regulate neuroblastoma growth, angiogenesis, and metastasis and is associated with dedifferentiation in *MYCN* amplified tumors.[80] Depending on the microenvironment, TAMs can become polarized into type 1 antitumor or type 2 pro-tumor phenotypes.[81] In the presence of anti-GD2 MAb, ADCC can transform pro-tumor M-CSF-activated macrophages into efficient antitumor effectors.[82]

Role of lymphokines

Patients with high risk neuroblastoma have severely compromised lymphoid systems (NK, T and B cells) because of dose-intensive induction therapy, at a time when antibody immunotherapy is applied. By activating ADCC to kill NB, anti-GD2 MAb is most efficient when effector cell populations and functions are amplified by cytokines. IL-2, which activates NK cells, natural killer T (NKT) cells, T-cells, and the undesirable regulatory T-cells (T_{reg}), have a modest anti-neuroblastoma effect as a single agent.[83] In contrast, the consistent ability of GM-CSF and G-CSF to repopulate and activate the myeloid system, and

their relative lack of toxicity, make them ideal cytokines to combine with anti-GD2 antibodies. Unlike GM-CSF, IL-2 is associated with substantial toxicity (e.g., 23% of patients experienced capillary leak[18]). Similar to IL-2, IL-15 activates NK, NKT, and CD8+ T-cells.[73] However, it does not cause capillary leak, activation-induced cell death, or increased T_{reg} activation in non-human primate studies.[84] Another type of anti-neuroblastoma lymphocytes called NKT cells can infiltrate neuroblastoma, kill TAMs, and they are associated with superior patient survival.[85] However, for NKT cells to survive the hypoxic neuroblastoma environment, IL-15 is essential.[86] When complexed to its IL-15Rα receptor, the biological effects of IL15 are enhanced.[87] IL15 fused to anti-GD2 antibody c. 60C3 displayed strong antitumor activities in syngeneic cancer models.[88] Preclinical evaluation of hu3F8 in combination with IL-15 has demonstrated similarly encouraging anti-tumor efficacy (unpublished data).

Compartmental efficacy of anti-GD2 antibodies

When the relapse pattern of patients following anti-GD2 immunotherapy was analyzed, it is striking to note that while diffuse relapse was no longer typical (only 34% of all relapses); isolated relapses in the BM or bone (27%), the brain parenchyma (23%), and soft tissues including nodes (16%) were increasingly frequent.[34] This suggests that intravenous anti-GD2 antibody may be more efficacious in the blood and marrow compartment than the CSF and lymphatic sites. Nonetheless, the marrow compartment remains an important reservoir for disease relapse since marrow MRD early after consolidation with immunotherapy was the strongest predictor of PFS and OS.[89]

Biochemical differences among the anti-GD2 antibodies

There are substantial biochemical differences among the anti-GD2 antibodies; however, whether they translate into differences in clinical efficacy remains to be proven. For example, 3F8 and hu3F8 have a much slower k_{off} (>10 fold) than 14G2a or ch14.18,[19] which could translate into longer retention time on the target. Although murine and chimeric antibodies (ch14.18) are usually immunogenic over time, neutralizing antibodies against humanized antibodies (hu3F8, hu14.18) can still occur in some patients. Proteins produced in SP2/0 cell lines versus CHO cell lines can be biochemically different. For example, 3F8 and ch14.18 were made in SP2/0 cell lines which add α-linked galactose or N-acetylneuraminic acid (NeuAc) epitopes not present in proteins made by human or by CHO cells (ch14.18-CHO, hu3F8).[90] High levels of natural anti-Gal[91] and anti-NeuGc antibodies[92] circulate in human blood, potentially capable to interfere with the pharmacokinetics of MAb, while causing immune complex disease or even anaphylactic reactions.[93,94]

Pain side effect, antibody dose, HAMA or HACA and MRD

GD2 is known to be expressed on neurons and peripheral nerves. CNS toxicity is rare partly because of the blood brain barrier to intravenous MAb. Moreover, anti-GD2 antibodies injected directly into the CSF space among patients with neuroblastoma and medulloblastoma have not produced any major long term CNS toxicity.[95] Nonetheless, the acute pain side effect of anti-GD2 antibody necessitates heavy doses of analgesics and sedatives with their complications requiring inpatient administration (e.g. ch14.18). Because

of pain, the dose of anti-GD2 is limited to 20–40 mg/m^2/day and usually for a maximum of 5 consecutive days. Without the option of dose escalation, it is not surprising the clinical benefit of anti-GD2 antibody is primarily seen in patients with MRD. Soft tissue bulky tumors generally do not respond. Various attempts to lessen the pain side effects have been tried. A phase I human trial was completed to test the effect of mutation (K322A) in the Fc region on reducing complement activation and the presumed pain side effect, but pain was still observed.[57] Premedication with a small dose of Fc-modified anti-GD2 antibody devoid of ADCC and CMC functions reduced significantly the doses of analgesics required, but pain side effects were not eliminated.[27] MAbs that target 9-O-acetyl-GD2 but not GD2 could potentially reduce pain toxicities associated with binding to GD2 antigen on peripheral nerves.[96] Although staff training and careful coordination in pain management has enabled safe outpatient administration (e.g. 3F8 and hu3F8), the identification of the novel receptor for this pain mechanism should provide a more rational and effective approach to reduce pain side effect and possibly to further dose escalate to improve anti-tumor effect.

Patients with HAMA have few to no pain side effects from MAb, as well as accelerated antibody clearance, and probably reduced or no therapeutic benefit. Hence, 3F8 treatments are postponed until HAMA falls below 1,000 U/mL. Unlike 3F8, HAMA and HACA are not part of treatment cycle prerequisite for 14G2a or ch14.18. How that translates into long term efficacy will require careful comparison. Interestingly, the ability to mount HAMA response (not the presence of HAMA during 3F8 administration) consistently correlated with long term overall survival for high risk neuroblastoma patients with stage 4 disease diagnosed after 18 months of age or with *MYCN* amplified tumors, irrespective if they were treated with primary refractory disease[97] or in their first remission.[34] We interpret this association as the result of active host anti-idiotypic network[60] or an induced anti-tumor response following opsonization of tumors,[82] responsible for the long term survival.

Antibody Drug Conjugates

Antibody immunoconjugates can selectively deliver the cytotoxic payload to the tumor. Such conjugates could be a drug (antibody drug conjugates, ADC) with known antitumor activity, radionuclide or toxin. There are several factors that can negatively affect clinical use of immunoconjugates. The conjugation chemistry can damage immunoreactivity, interfering with antibody binding to the target. Instability of the conjugate in serum can nullify its effect meant for the target, sometimes resulting in unintended systemic toxicities. Release of the active moiety at the tumor target can also be impaired by the microenvironment. The development of anti-conjugate antibodies represents another potential obstacle for immunoconjugates. Despite the success of antibody-toxin conjugates (Gemtuzumab ozogamicin) in AML and antibody-microtubule-poisons (Brentuximab Vedotin in Hodgkin's lymphoma or Trastuzumab Emtansine in breast cancer), ADC targeting GD2 is in the exploratory phase in preclinical testing.[98]

Radionuclide immunoconjugates for radioimmunotherapy (RIT)

Radionuclides can exert antitumor effect not just on antigen(+) but also on surrounding antigen(−) tumor cells via a “cross-fire” effect and radiation-induced intercellular signaling.[99]

Intravenous anti-GD2 ^{131}I-3F8 has been tested in children with metastatic neuroblastoma at high radioisotope doses (6–28 mCi/kg).[100] Although responses were seen in both soft tissue masses and bone marrows, the use of myeloablative ^{131}I-3F8 (20 mCi/kg) did not add survival benefit compared to naked 3F8 among patients with high risk metastatic stage 4 neuroblastoma in first remission.[34] In a subsequent phase I trial of ^{131}I-3F8 in combination with bevacizumab (NCT00450827) in patients with relapsed or refractory NB, responses were similar to those with ^{131}I-3F8 alone.

Intraventricular ^{131}I-3F8 – compartmental RIT (cRIT)

While intravenous radiolabeled 3F8 did not improve survival, results with cRIT are highly promising. Brain metastasis is a common pathway for metastatic solid tumors; its biology is poorly understood, management inadequate, and cure is rare. Except for occasional tumor types, most patients with brain metastases die despite intensive therapy. Tumor cells from blood or from brain metastases can invade the CSF and disseminate throughout the neuroaxis by the constant flow of CSF from the ventricles to the spinal canal and over the cortical convexities, a condition called leptomeningeal (LM) carcinomatosis with significant morbidity and mortality. In contrast to leukemia, where LM metastasis is well controlled by intrathecal chemotherapy, the prognosis in solid tumors is extremely guarded despite the use of chemotherapy and radiation therapy. Neuroblastoma metastasis to the CNS was once considered rare. This entity is distinguishable from dural based metastases where direct extension from bony disease is the rule. As the natural history of neuroblastoma changed with systemic immunotherapy,[22] isolated CNS afflicts up to 20% of the neuroblastoma patients at Memorial Sloan-Kettering Cancer Center (MSKCC), where their systemic disease was thought to have been eradicated.[34] Conventional treatment modalities, including surgical resection, chemotherapy and radiation, have not improved survival.[101]

The CSF (thecal sac) space has unique characteristics suitable for cRIT: (1) the blood brain barrier prevents MAb recirculation, (2) compared to blood, CSF has few white cells, no Fc receptors (FcR), and ~1000-fold less IgG.[102] MAb injected into the CSF compartment is better shielded from host immunity with less sequestration by FcR or degradation by enzymes, (3) the 200-fold lower protein content of CSF (versus serum) facilitates MAb binding, (4) since CSF volume is small (140 ml), MAb achieves a very high compartmental concentration, (5) CSF compartment is renewed every 7–8 hours, providing a built-in washing step, (6) CSF flow can be reduced pharmacologically, permitting longer MAb reaction time, (7) the apparent absence of anatomic barrier facilitates the movement of MAb between CSF and the extracellular space of brain, especially if there is damage to the meninges either by tumor itself or after surgical resection of tumor.

Intraventricular ^{131}I-3F8[103] when tested in cRIT for leptomeningeal cancers in both children and adults showed highly favorable therapeutic ratios. Toxicities included self-limited

headache, fever, and vomiting. DLT was reached at the 20-mCi dose, when transient elevations in intracranial pressure and chemical meningitis were seen. Three of 13 assessable patients in the phase I study achieved objective radiographic and/or cytologic responses. No late toxicities have been seen in two patients who remain in remission off therapy for nearly 10 years. Among children with recurrent neuroblastoma metastatic to the CNS, when cRIT was incorporated into multi-modality therapies, long term remissions have been achieved (NCT00445965).[104]

^{131}I-3F8 cRIT for relapsed medulloblastoma (MB, NCT00445965)[95]

Recurrent and high risk MB is challenging to cure. 36 patients with high risk (n=5) or recurrent (n=31) MB were treated on MSKCC IRB-approved protocol. Patients had <72 Gy (brain parenchyma) and 45 Gy (spinal cord) prior radiation. Patients received 2 mCi ^{131}I-3F8 followed by 2–4 weekly injections (10 mCi ^{131}I-3F8/injection), maximum CSF dose 2400 cGy, determined by CSF samplings and region of interest analyses on whole-body scintigraphy. 30 patients received >2 therapeutic injections. 32/36 (89%) remained alive ≥6 months after cRIT. Long term survivors in CR included 10 of 19 (53%) patients treated with ^{131}I-3F8 as consolidative therapy, at a mean follow up of 49.6 months (6 months to 6 years); 4 of 17 (24%) with radiographic evidence of relapsed or refractory MB remain alive, 2 in CR 3.5 yrs and 5.5 yrs since relapse. Four of 5 patients with high risk MB remain in CR (mean follow up 54.4 months); 1 patient died of secondary glioma 5.3 years after initial diagnosis. Mean total absorbed CSF dose was 1250 cGy (237 – 2239) by sampling; average CSF dose was 64.1 cGy/mCi and blood was 2.9 cGy/mCi. Toxicities included self-limited headache, fever, and vomiting. No long term side effects directly attributable to ^{131}I-3F8 have been observed.

Lessons learned from clinical application of radioiodinated 3F8

Choice of radioisotope and immunoreactivity

Most clinical applications of RIT utilize β-emitting radioimmunoconjugates. β-particles have a relatively long range (0.8–5 mm) and low linear energy transfer (approximately 0.2 keV/μm). This long range results in the delivery of radiation not only to the antigen-positive, but also to antigen-negative tumor cells, as well as to the surrounding normal tissues. Thus, β-emitters can treat bulky diseases effectively, but are not optimal for eradicating single cells or micrometastasis. Most early human studies of RIT used iodine 131 (^{131}I), a long-lived β-particle emitter. Because of its γ-particle emission, it is also suitable for dosimetry studies. However, γ-particle emission poses a radio-hazard at high treatment doses, necessitating patient isolation. In vivo dehalogenation can compromise tumor dose with subsequent thyroid damage from the released iodide. Yttrium 90 (^{90}Y) is a pure β-emitter; its lack of γ-radiation allows outpatient treatment. However, ^{90}Y has its limitations, including deposition in bone when dissociated from the MAb complex and its lack of γ-emissions, thereby requiring the use of indium 111 (^{111}In) to estimate biodistribution and dosimetry. Lutetium-177 (^{177}Lu), also a pure β-emitter, has a longer half-life of 6.6 days, more compatible with biologic half-life of MAb than ^{90}Y (2.6 days). RIT with ^{177}Lu may result in lower off-target radiation doses to surrounding normal tissues, compared with ^{90}Y and ^{131}I especially when multistep targeting (MST) strategies are used (see below).[105]

α-particles are helium nuclei; when compared with β-particles, they have a shorter range (50–80 μm) and a higher linear energy transfer (approximately 100 keV/μm). As few as one or two α-particles can destroy a target cell. RIT using α-emitters should result in less nonspecific toxicity to normal bystanders as well as more efficient single cell killing. This is ideal for controlling MRD. α-particle-emitting isotopes such as astatine-211 (^{211}At, T½=7.2h, 1 α particle), bismuth-213 (^{213}Bi, T½=46 min, 1 α particle), and actinium-225 (^{225}Ac, T½=10d, 4 α particles) have proven safe in clinical trials.[106] The relative lack of extramedullary toxicities should encourage further development of this targeting technique for micrometastases or neoplasms on the surface of body compartments, such as ovarian cancer and leptomeningeal metastasis.[107,108] Radium-223 (^{223}Ra, T½=11.4 d, 4 α particles) is now FDA approved for prostate cancer, and lead-212 (^{212}Pb, T½=10.6h, 1 α particle) is another nanogenerator (giving rise to ^{212}Bi, T½=25 min, 1 α particle), both are potential candidates for MAb targeted RIT. Modeling studies of ^{131}I-3F8 cRIT clearly suggests that improving immunoreactivity from the current 50%[19] to 90%[109] could potentially bring about a major gain in the therapeutic index. MST offers an opportunity to maintain unimpaired immunoreactivity in the first step. Site directed conjugation using glycolinks and click chemistry present exciting alternative approaches. With the optimal choice of isotopes, skillful conjugation methods, RIT and cRIT could become important in the management of hard-to-cure neuroblastoma and other GD2(+) cancers.

Dosimetry using Theranostic agents

Quantitative methods for estimating radiation absorbed dose are now feasible and they are essential for individualizing patient treatment and avoiding excessive radiation toxicities. As theranostics, a radiolabeled MAb in tracer amounts serves as a diagnostics/dosimetry agent, and if calculations are favorable, can be given as a scale up for therapeutic benefit. Quantitative high resolution PET/CT imaging of antibodies (e.g. positron emitting ^{124}I-3F8) provides accurate dosimetry and staging information. Since the dosimetry of positrons (β+) from ^{124}I are similar to electrons (β−) from ^{131}I, ^{124}I labeled MAb can now be used as theranostic agents, e.g. in brain stem glioma (NCT01502917). Using these theranostic setups, patient and tumor selection will provide a rational basis for achieving durable therapeutic effects.

cRIT optimization[110]

Pharmacokinetic models have been used to evaluate the role of kinetics and transport parameters of cRIT in maximizing the therapeutic ratio, the ratio of area under the curve (AUC) for the concentration of the bound antibodies over time when compared to that for unbound antibodies. Using a single compartment model, it was predicted that increasing the affinity of antibodies to antigens greatly increased AUC for the same amount of isotope administered; smaller antibody dose and higher specific activity also improved therapeutic ratio. When the isotope half-life was 0.77 h, increasing the antibody association constant enhanced AUC much more than did decreasing the dissociation constant, even if overall affinity was unchanged. When isotope half-life reached 240 h, decreasing the dissociation constant would slightly enhance AUC. Other predictions were that decreasing the CSF bulk flow rate would increase AUC, with 3 mL/h being optimal; continuous infusion or split dose

antibody can improve AUC by 1.8 fold; for antibody affinity of 10 nM, choosing an isotope with half-life of 64 h (instead of 0.77 h) could greatly enhance the therapeutic ratio.

In a subsequent two compartment model,[109] fitting to patient data was improved when compared to the one-compartment model (R=0.92±0.11 versus 0.77±0.21, p=0.005). In addition, the following new predictions were made: (1) Increasing immunoreactivity of ^{131}I-3F8 from 10% to 90% increased both AUC and therapeutic ratio by 7.4 fold, (2) When extrapolated to the clinical setting, the model predicted that if ^{131}I-3F8 could be split into 4 doses of 1.4 mg each and given at ≥24 hours apart, an antibody affinity of 4nM at 50% immunoreactivity was adequate to deliver ≥100 Gy to tumor cells while keeping normal CSF exposure to <10 Gy.

Multistep targeting or pretargeting[111,112]

Suboptimal tumor radioactivity AUC versus blood or kidney AUC ratios (~3:1) has been the major limiting factor for RIT. cRIT took advantage of the fast clearance of unbound ^{131}I-3F8 (half life of 3–12 h) out of the CSF, in contrast to 2–4 days for IgG in the blood. A promising solution to improve this therapeutic ratio uses a multistep procedure that pretargets the antibody before the binding of the cytotoxic ligand to the tumor. Generally, a tumor-specific antibody is conjugated to a ligand-binder, such as streptavidin (with high affinity for biotin)[113] or ligand-specific antibody (binding to metal chelators, such as diethylene triamine pentaacetic acid [DTPA] or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid [DOTA]).[105,112] In the first step, these bispecific antibodies (172–200 kD) are allowed to localize to tumors in vivo, and any excess is cleared from the blood. The small radiolabeled ligand (DOTA-radiometal or its biotinylated form) is then injected intravenously. The ligand penetrates tissues rapidly and by virtue of the high affinity interaction, binds tightly to the antibody-conjugate at the tumor site. Unbound ligand is quickly excreted through the kidneys. Because of the short transit time of the toxic ligand (radionuclides or toxins), a substantial improvement in the therapeutic ratio is achievable without sacrificing the percent injected dose per gram in tumor.[105] However, MST using streptavidin has been limited by immunogenicity, the absence of a clinical clearing agent, difficulty in manufacture and purification, interfering substances in human blood, and the nonversatility of the “hook” in terms of affinity variants. The modular (IgG-scFv) antibody form,[105] with the IgG portion specific for tumor and the high affinity scFv (C825) specific for DOTA-metal has provided novel solutions. These bispecific bivalent constructs have high avidity for both the tumor and DOTA. The large molecular weight (~200 kD) ensures a long plasma half-life for optimal tumor targeting. More importantly, since the scFv affinity for DOTA depends on the chelated metal, ranging from 8 pM to 50 nM affinity, dextrans carrying DOTA-metal of low affinity for scFv can be exploited as clearing agents. Besides targeting ^{90}Y (11 pM affinity for scFv) or ^{177}Lu (11 pM affinity for scFv) in RIT, DOTA-metal provides a convenient hook to target nanoparticles. The proof of concept in animal studies was recently demonstrated using the hu3F8-C825 bispecific construct achieving a >20 AUC ratio for kidney and >100 ratio for blood, curing neuroblastoma without clinical or histologic toxicities.[112]

Emerging therapeutics targeting GD2

Nanoparticles[98]

Inorganic nanoparticles are delivery systems built to precise specifications for medical applications. They represent small particles (3–200 nm) made up of inert scaffolds. As therapeutics, they are built for their superior pharmacokinetics, thereby enhancing intracellular concentration of drugs in cancer cells while minimizing their systemic side effects. In neuroblastoma-bearing mice, fenretinide, a synthetic retinoic acid derivative,[114] or doxorubin[115] can be encapsulated in immunoliposomes targeted by 14G2a/ch14.18 anti-GD2 antibodies to completely inhibit the development of macroscopic and microscopic metastases. Antisense oligonucleotides can also be encapsulated in such liposomes to downregulate tumor associated gene expression, increase cytokines production and stimulate innate immune system (macrophages and NK cells).[116] Ch14.18 conjugated to the surface of porous silica nanoparticles containing microRNA inhibited neuroblastoma xenograft growth,[117] where miR-34a activates a caspase-mediated apoptotic pathway.[118] It also targets multiple genetic pathways associated with oncogenesis including *MYCN, BCL2, NOTCH1, JAG1, CCND1, CDK6, E2F3* and others.[119,120] *ALK*-siRNA embedded in such liposomes can also inhibit neuroblastoma growth in animal models.[121] Gold nanorods targeted by anti-GD2 antibodies induced thermolysis of neuroblastoma by releasing heat in the nanoenvironment when exposed to near infrared laser light – a process called thermoablation.[122] Similar effects have been observed in anti-GD2-targeted carbon nanotubes.[123]

Chimeric Antigen Receptors (CARs)

T-cell mediated immunity can be extremely effective for cancer therapy if these highly potent effector cells can be harnessed, as shown in multiple preclinical studies and clinical trials. This potential was recently realized when polyclonal T-cell tumoricidal activity was redirected to surface TAA. Human cancers evade the classic T cell surveillance through a number of well established mechanisms, the most important one being the downregulation of MHC and co-stimulatory molecules. Tumor-specific CARs enable the recognition of TAA by T-lymphocytes in an HLA-independent manner. Introduction of co-stimulatory molecules (CD28, 4-1BB, OX40) provides essential “second signal” for further activation of T-cells and therefore obviates the need for “external” co-stimulation.[124,125] To date, most clinical studies have been conducted in patients with leukemia, neuroblastoma, mesothelioma and glioblastoma multiforme. Attempts have been made to improve the kinetics of CAR-grafted cells, including their persistence and proliferation in vivo. To add a second stimulatory signal (through endogenous EBV virus), EBV-specific T-cells were used to make GD2-CAR modified T cells.[126] In this seminal study, 4 of the 8 patients with evaluable tumors had evidence of tumor necrosis or regressions, including a complete remission. Importantly, no major side effects of immunotherapy were noted in this study. None of the patients had detectable antibody response to CAR-grafted T-cells. Impressive results were reported by the same group in 19 patients with high-risk neuroblastoma.[127] Three of 11 patients with active disease at the time of treatment achieved complete remission. Interestingly, superior clinical outcome was associated with persistence of CAR-modified T-cells beyond 6 weeks after infusion. Encouraging results with GD2-specific

CARs has prompted further clinical investigations using third generation CARs (NCT01822652), where the anti-GD2 scFv is fused with 4-1BB or CD28 in tandem with CD3ζ. The transgene used in this study also contains icaspase9 suicide switch that can be activated in case the transduced T cells need to be sacrificed. Another phase I study using VZV-specific CARs is also planned (NCT01953900). Efficacy of immunotherapy with GD2-redirected CARs in other than neuroblastoma GD2-positive tumors is being tested in preclinical studies.[128] Besides T cells, NK modified to express GD2-CAR could overcome NK resistance.[129] Feasibility and safety of allogeneic haploidentical NK cells administered together with hu3F8 in patients is planned.

Bispecific antibodies (BsAb) to engage T cells

The potential of exploiting cytotoxic potential of T-cells in conjunction with GD2 specific antibodies has led to the development of bispecific T-cell engagers (BiTE). Standard MAb are IgGs that kill by Fc-dependent mechanisms such as CMC and ADCC. T-lymphocytes have no Fc receptors, and are therefore under-utilized during standard MAb therapy. Moreover, with few exceptions, T cells are inefficient or incapable of targeting carbohydrate epitopes. BsAb recruit T cells for tumor cytotoxicity through HLA-non-restricted CD3-mediated activation. By engaging polyclonal T cells BsAb can overcome the low clonal frequency of classic T cell mediated anti-tumor immunity. CD3 engagement can also induce T cell proliferation and generation of effector cytokines to potentiate the anti-tumor effect. BiTEs, which are based on single-chain antibodies, can exhibit multiple rounds of target cell lysis by T cells.[130] Clinical efficacy of low dose blinatumomab, a BiTE with dual specificity for CD19 and CD3, was highly encouraging among patients with relapsed non–Hodgkin's B cell lymphoma.[131] Anti-GD2 BsAb can kill a wide spectrum of GD2(+) tumors with femtomolar EC50 in the presence of expanded human T-cells, a potency orders of magnitude stronger than the nanomolar or picomolar EC50 for IgG mediated ADCC or CMC.[132,133] GD2/CD3 BsAbs have demonstrated high efficiency in tumor ablation in xenograft models.[132–134] Both tandem scFv format (BiTE)[132] as well as IgG-scFv modular formats[133] have shown anti-tumor efficacy. Murine anti-GD2 antibody ME36.1 has also been used to engineer a trifunctional antibody TRBS07,[135] a quadroma hybrid antibody with monovalent binding to GD2 and CD3, carrying a specially engineered chimeric mouse IgG2a x rat IgG2b Fc region with preferential binding to activating FcγR expressed on monocytes, macrophages, dendritic and natural killer cells.[135] In mice models, it induced a polyvalent T-cell response and a vaccination effect.

Future directions

Anti-GD2 antibodies have been extensively evaluated in preclinical and clinical studies in the past two decades. Therapeutic efficacy and acceptable safety profile have made them an integral part of the current treatment of neuroblastoma. Curing high risk metastatic neuroblastoma diagnosed ≥18 months of age or with *MYCN* amplification was unthinkable 25 years ago. The addition of GD2-targeted treatments has greatly improved their outlook, where >50% progression-free survival beyond 5 years is now possible. Several key properties of antibodies and principles of therapeutic applications have been identified, improved, or to be optimized. These have encompassed antibody affinity, novel antibody

forms including CAR and BsAb, multistep targeting, choice of isotopes, dosimetry, addition of novel cytokines, and alleviation of the pain side effects. With more effective delivery and less pain side effects, eradicating GD2-positive soft tissue tumors beyond the MRD setting may be possible. Retargeting T cells using CAR or BsAb holds great promise especially for tumors that are less sensitive to Fc-dependent cytotoxicities (ADCC or CMC). Importantly, these redirected T-cells can obviate tumor immune evasion, such as the down-regulation of HLA and co-stimulatory molecules. Although strategies are in place to reduce adverse cytokine storm, careful dosing and schedule will need to be tested in the upcoming clinical trials. Since GD2 is expressed on a wide spectrum of human tumors, including osteosarcoma, soft tissue sarcomas, SCLC, melanoma and retinoblastoma, GD2-targeted strategies could be beneficial in these other tumor types.

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Table 1

Clinical Trials of Murine Anti-GD2 Antibodies.

Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Best Response	Long Term Survival (5 years from start of Mab therapy)	Important observations
IgG3 3F8	−/[24][26]	Phase I	3F8 antibody in patients with neuroblastoma (8) and melanoma (9)	5–100 (single dose per cycle), 1 cycle	hypertension (dose-limiting), fever, pain, urticaria	2 CR (2 PR when reassessed using INRC)	Not reported	Specific binding to tumor; correlation between HAMA level, side effects and response
IgG3 3F8 and heat-modified 3F8	NCT00450307/[27]	Phase I	3F8 antibody dose escalation plus GM-CSF in high-risk neuroblastoma (30)	20–160 (x 5 days per cycle), 2 cycles	fever, pain, urticaria, hypertension	3 CR 2 PR	Not reported	Heat-modified 3F8 antibodies decrease toxicity without blunting anti-NB activity
IgG3 3F8	NCT00037011/[37]	Phase I	3F8 and barley-derived-β-D-glucan in chemoresistant neuroblastoma (24)	10 (x 10 days per cycle), upto 4 cycles	pain, fever, urticaria, thrombocytopenia	MIBG response 13/22	Not reported	No DLT for barley glucan up to 80 mg/kg/dayx1 0 days; antineoplastic activity in advanced-stage neuroblastoma
IgG3 3F8	NCT00492167/[38]	Phase I	3F8 and yeast-derived-β-D-glucan in chemoresistant neuroblastoma (44)	10 (x 10 days per cycle)	pain, fever, urticaria	MIBG response: 7/24 CR of BM: 3/5 Overall response: 1 CR, 1 PR, 22 SD, 17 PD	Not reported	No DLT for yeast glucan up to 160 mg/kg/day x17 days; antineoplastic activity in advanced-stage neuroblastoma
IgG2a (14.G2a)	−/[40]	Phase I	prolonged iv infusion of 14G2a antibody in patients with neuroectodermal tumors (18)	10–40 (x 5 days per cycle), 1–3 cycles	pain, hyponatremia, fever, rash, paresthesias, weakness, chronic hypotension	2/5 PR in neuroblastoma	Not reported	modest antitumor activity at the expense of significant toxicity
IgG2a (14.G2a)	−/[41]	Phase I	14.G2a antibody plus interleukin-2 in GD2+ tumors (33)	2–60 (x 5 days per cycle), 1–3 cycles	pain, fever, rash, allergic or anaphylactic reactions	1/31 PR in neuroblastoma, 1/2 CR in osteosarcoma	Not reported	Rare antitumor effect in bulky disease
IgG3 3F8	−/[28, 35]	Phase II	3F8 antibody alone in treatment of minimal residual stage 4	10 (x 10 days per cycle) upto 4 cycles	pain, fever, urticaria, reversible decreases in blood counts	MIBG response 6/16 CR of BM (immunocytology) 6/9 CR pf MRD 7/12	For patients treated in 1st remission:	long-term remission without autologous BMT can be achieved with 3F8; transient HAMA associated with

Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Best Response	Long Term Survival (5 years from start of Mab therapy)	Important observations
			neuroblastoma (34) neuroblastoma (34)				PFS at 5y = 44% OS at 5y = 49%	significantly better survival
IgG3 3F8	NCT00002560/[30]	Phase II	3F8 antibody and iv GM-CSF in refractory neuroblastoma (45)	10 (x 10 days per cycle), q4–8 weeks up to 2 years (range 1–14)	Pain, rash	CR of BM 12/19 (primary refractory), 5/10 (secondary refractory) neuroblastoma	PFS at 5y (primary refractory) = 11%; OS at 5y = 17%	3F8/GM-CSF very effective against chemoresistant primary refractory neuroblastoma in bone marrow, but not PD or soft tissue masses
IgG3 3F8	NCT00072358/[33]	Phase II	3F8 antibody and sc GM-CSF in high risk primary refractory stage 4 neuroblastoma (79)	20 (x 5 days per cycle), q1–3 months to 2 years (mean 7.7, median 7, range 1–25 cycles)	Pain, fever, urticaria, hyperension, hypoxia, line infection, transient ALT/AST elevations, reversible decreases in WBC	CR of BM: 40/46 (87%) CR of MBIG: 10/26 (38%)	PFS at 5 y = 24% OS at 5y = 65%	***Favorable*** prognostic markers: HAMA, FCGR2A high affinity allele ***Adverse*** prognostic markers: postive MRD after 2 cycles of 3F8/ GMCSF ***Not prognostic***: *MYCN* amplifcation, prior stem cell transplant
IgG3 3F8	NCT00002560/[34]	Phase II	3F8 antibody and iv GM-CSF in high risk stage 4 neuroblastoma in first remission (41)	20 (x 5 days per cycle), q1–3 months to 2 years (mean 4.6, median 4, range 1–13 cycles)	Pain, fever, urticaria, hyperension, hypoxia, line infection, transient ALT/AST elevations, reversible decreases in WBC	NA	PFS at 5y = 56% OS at 5y = 61%	Relapse mostly at isolated sites (CNS, soft issue and marrow/bone). ***Favorable*** prognostic factors: missing ligand for inhibitory killer immunoglobulin-like receptor (KIR), HAMA, and scGM-CSF.
IgG3 3F8	NCT00072358/[34]	Phase II	3F8 antibody and sc GM-CSF in high risk (57) and ultra high risk (28) stage 4 neuroblastoma in first remission	20 (x 5 days per cycle), q1–3 months to 2 years (mean 6.4, median 6, range 1–14 cycles)	Pain, fever, urticaria, hyperension, hypoxia, line infection, transient ALT/AST elevations, reversible decreases in WBC	NA	HR: PFS at 5y = 62%, OS at 5y = 81%; UHR PF at 5 y = 36%, OS at 5 y = 75%	***Adverse*** prognostic factors: difficulty getting into first remission, MRD(+) after 2 cycles of immunotherapy. ***Not prognostic***: *MYCN* amplification, prior stem cell transplant
IgG3 3F8	NCT00072358/[35]	Phase II	3F8 antibody and sc GM-CSF in high risk stage 4	20 (x 5 days per cycle), q1–3 months to 2 years (mean 4.4,	Pain, fever, urticaria, hyperension, hypoxia, line	NA	PFS at 4 y = 33% OS at 5y = 53%	Long-term PFS and OS are possible for after relapse if patients

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Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Best Response	Long Term Survival (5 years from start of Mab therapy)	Important observations
			neuroblastoma in second remission (101) neuroblastoma in second remission (101) neuroblastoma in second remission (101)	1–12 cycles	infection, transient ALT/AST elevations, reversible decreases in WBC			achieve >=2nd CR/ VGPR ***Favorable*** prognostic variables: ≤18 months at diagnosis, maintenance chemotherapy, rituximab for HAMA ***Unfavorable*** prognostic marker: MRD(+) after 2 cycles of immunotherapy

Table 2

Clinical Trials of Chimeric Anti-GD2 Antibodies.

Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Response	Important observations
Ch14.18	−/[45]	Phase I	ch14.18 in patients with malignant melanoma (13)	5–100, 1–2 days	pain	No response	antibody response directed at the variable region of ch14.18
Ch14.18	−/[46]	Phase I	ch14.18 in patients with neuroblastoma (9)	30–50, daily for 5 days, 1–4 courses	pain, fever, allergic reactions, ocular symptoms	2 CR, 2 PR, 1 MR	ocular toxicity, no antibody response to ch14.18
Ch14.18	−/[47]	Phase I	ch14.18 in patients with refractory neuroblastoma (10) and osteosarcoma (1)	10–200, daily for 1–4 days, 1–5 courses	Pain, fever, hypertension, hypotension, serum sickness, rash	1 PR, 4 MR	7/11 developed antibody response to ch14.18; all patients with high titers of anti-chl4.18 had therapeutic benefits
Ch14.18	−/**[48],** [49]	retrospective	consolidation treatment with ch14.18 in children > 1 year with metastatic neuroblastoma (166)	20, daily for 5 days, 6 cycles	fever, abnormal C-reactive protein, cough, rash, pain, ocular symptoms, capillary leak syndrome	Improved OS in antibody – treated group	no advantage of antibody treatment for EFS and OS in multivariate analysis; in followup study: ch14.18 may prevent late relapses
Ch14.18	−/[50]	Phase I	ch14.18 with GM-CSF in children with neuroblastoma after hematopoietic stem-cell transplantation (19)	20–50, daily for 4 days, up to 6 cycles	pain, fever, nausea, vomiting, urticaria, hypotension, capillary leak syndrome, neurotoxicity	No response	HACA detected in 5/18 patients (28%); development of HACA response not noted until after the second course
Ch14.18	−/[51]	Phase II	chimeric anti-GD2 (ch14.18) and GM-CSF for refractory neuroblastoma (32)	50, daily for 5 days	pain, fever, nausea, vomiting, diarrhea, urticaria, hypotension, hypertension, thrombocytopenia	1 CR, 3 PR, 1 MR	anti-tumor response to ch14.18 and GM-CSF combination in refractory disease
Ch14.18	−/[52]	Phase I	ch14.18 and rhGM-CSF in metastatic melanoma (16)	15–60, day 1	pain, blood pressure changes, nausea, diarrhea, peripheral nerve	No response	toxicity similar to that observed with ch14.18 alone without

Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Response	Important observations
					dysesthesias, myalgias, and weakness		improvement in tumor response
					dysesthesias, myalgias, and weakness		
					dysesthesias, myalgias, and weakness		
Ch14.18	−/[53]	Phase I	Ch14.18 plus IL-2 in melanoma patients (24)	2–10, daily for 5 days, 1–2 courses	allergic reactions, weakness, pericardial effusion, decreased performance status	1 CR, 1PR	Induced immune activation in all patients
Ch14.18/CHO	**NCT01704872/**[54]	Phase I	Ch14.18 antibody produced in CHO cells in relapsed or refractory Stage 4 neuroblastoma patients: a SIOPEN Phase 1 study (16)	10–30, daily for 5 days, 1–3 courses	similar to side effects in studies with ch14.18/ SP2/0	2 PR	in mice, comparable pharmacokinetics and biodistribution between ch14.18/CHO and ch14.18/SP2/0; similar in human (no direct comparison)
Ch14.18/CHO	−/[55]	not specified	Ch14.18 antibody produced in CHO cells with sc IL-2 in high risk neuroblastoma patients (53)	100, continous infusion for 10 days	not specified	overall response following INRG guide lines − 30%.	patients with KIR/KIRL mismatch and high affinity FCGR alleles showed a trend towards longer EFS (P= 0.08)
Ch14.18+R24	−/[56]	Phase I	Phase I trial of combined treatment with ch14.18 and R24 MAbs and IL-2 inpatients with melanoma and sarcoma (27)	2–7.5 (ch14.18) and 1–10 (R24), daily for 5 days, 1–3 courses	severe allergic reactions, pain	2 PR	MTD of ch14.18 and of R24 were lower than the MTD of each antibody
Ch14.18	**NCT00026312/**[18]	Randomized phase III	Ch14.18 with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma (113)	25, daily for 4 days, 5 cycles	Pain, capillary leak syndrome, hypersensitivity reactions	superior EFS and OS in immunotherapy group as compared to standard therapy	ch14.18, GM-CSF, and interleukin-2 associated with a significantly improved outcome as compared with standard therapy in patients with high-risk neuroblastoma
hu14.18	**NCT00743496 [**57]	Phase I	humanized 14.18 antibody with a single point mutation (K322A) that	2 to 70 mg/m2 dayly for 4 days every 28 days	pain, fever, 4CR, cough, asthenia, sensory neuropathy, anorexia, serum	2 PR	recommended phase II dose 60 mg/m2/day for 4 days, adverse effects were manageable and improved with subsequent courses

Type of antibody	ClinicalTrials.gov identifier/reference	Study type	Type of therapy (number of patients)	Dose, mg/m^2/day	Toxicity profile	Response	Important observations
			reduces complement-dependent lysis (36)		sickness,		
			reduces complement-dependent lysis (36)		hypertensive		
			reduces complement-dependent lysis (36)		encephalopathy		
			reduces complement-dependent lysis (36)				

Abbreviations: CR = complete response; PR = partial response; MR = mixed response; GM-CSF = granulocyte-macrophage colony-stimulating factor; HACA = human anti-chimeric antibody; HAMA = human anti-mouse antibody; iv = intravenous; sc = subcutaneous; MR = minor response, EFS = event-free survival

Table 3

Binding kinetics of mouse 3F8 and hu3F8 antibodies to human FcγRII (CD32)

Antibody		kon (1/Ms)	SD	koff (1/s)	SD	KA=kon/koff	koff (relative to CD32A-131R)	KA (relative to CD32A-131R)
mouse 3F8 (IgG3)	CD32A-131R	2.59E+04	9.31E+03	3.63E-01	2.36E-02	7.15E+04	1	1
	CD32A-131H	1.90E+04	3.58E+03	1.10E+00	8.27E-02	1.73E+04	3.02	0.24
	CD32B	1.15E+04	1.60E+03	2.03E+00	3.78E-01	5.66E+03	5.6	0.08
hu3F8 (IgG1)	CD32A-131R	1.49E+05	4.38E+04	4.06E-01	4.94E-02	3.68E+05	1.12	5.15
	CD32A-131H	2.70E+05	9.48E+03	4.27E-01	3.59E-02	6.31E+05	1.18	8.83
	CD32B	2.86E+04	1.63E+04	3.41E-01	2.63E-01	8.37E+04	0.94	1.17

*Kinetics of FcγRII (CD32) interaction with mouse 3F8 and hu3F8 were measured using a Biacore T100 biosensor and CM5 sensor chips (Biacore AB of GE Healthcare, Uppsala, Sweden). Toward the CD32A-131R allele, 3F8 had 2.67 fold slower off rate (koff), and nearly 3-fold higher binding affinity (Ka) than toward CD32A-131H. For hu3F8, the high affinity allele was 131-H. Affinity for CD32B was much lower for mouse 3F8 than for hu3F8.