Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012

Cancer Therapy: Clinical

Clinical Cancer Research

# Phase I Trial of a Bivalent Gangliosides Vaccine in Combination with β-Glucan for High-Risk Neuroblastoma in Second or Later Remission

Brian H. Kushner[1], Irene Y. Cheung[1], Shakeel Modak[1], Kim Kramer[1], Govind Ragupathi[2], and Nai-Kong V. Cheung[1]

## Abstract

**Purpose:** To report on a phase I trial designed to find the maximally tolerated dose in children of the immunologic adjuvant OPT-821 in a vaccine containing neuroblastoma-associated antigens (GD2 and GD3; Clinicaltrials.gov NCT00911560). Secondary objectives were to obtain preliminary data on immune response and activity against minimal residual disease (MRD). Treatment also included the immunostimulant β-glucan.

**Experimental Design:** Patients with neuroblastoma in ≥2nd complete/very good partial remission received vaccine subcutaneously (weeks 1–2–3–8–20–32–52). Vaccine contained 30 μg each of GD2 and GD3 stabilized as lactones and conjugated to the immunologic carrier protein keyhole limpet hemocyanin; and OPT-821, which was dose escalated as 50, 75, 100, and 150 μg/m$^2$ per injection. Oral β-glucan (40 mg/kg/day, 14 days on/14 days off) started week 6.

**Results:** The study was completed with 15 patients because there was no dose-limiting toxicity at 150 μg/m$^2$ of OPT-821 (the dosing used in adults). Thirteen of fifteen patients received the entire protocol treatment, including 12 who remain relapse-free at 24+ to 39+ (median 32+) months and 1 who relapsed (single node) at 21 months. Relapse-free survival was 80% ± 10% at 24 months. Vaccine and β-glucan were well tolerated. Twelve of fifteen patients had antibody responses against GD2 and/or GD3. Disappearance of MRD was documented in 6 of 10 patients assessable for response.

**Conclusions:** This immunotherapy program lacks major toxicity and is transportable to any outpatient clinic. Patient outcome is encouraging but the efficacy is uncertain because of the complexity and heterogeneity of prior therapies. A larger phase II trial is underway. *Clin Cancer Res; 20(5); 1375–82. ©2014 AACR.*

## Introduction

Relapse of high-risk neuroblastoma (HR-NB) has long been viewed as tantamount to eventual death from progressive disease or toxicity (1). We hypothesize that active humoral immunity could maintain second or subsequent remission even among these ultra-high-risk patients. This curative approach starts from diagnosis and has several components. First, patients in initial remission undergo close monitoring, with extent-of-disease evaluations at least every 3 months through ≥3 years from diagnosis; the aim is to detect focal relapse early, which may be more amenable to successful salvage than a widespread relapse with a large tumor burden (2). Second, relapse is treated with a multimodality program that comprises surgery and/or radiotherapy for local control, plus salvage chemotherapy combining agents with well-established anti-neuroblastoma activity and tailored to the patient's particular clinical context or past treatment history (3–8). Third, after achieving a maximal response with the standard modalities (chemotherapy, radiotherapy, surgery), remission is consolidated via immunotherapy using monoclonal antibody (MoAb) or vaccine, the latter being the subject of this report.

The vaccine is designed to induce antibodies against gangliosides GD2 and GD3, which are highly expressed on neuroblastoma. Indeed, the intensity of ganglioside expression on neuroblastoma is unique. Although melanoma (considered a ganglioside-rich tumor) yields a median of 43 mcg of GD3 per gram of tissue biopsied and 3.1 mcg of GD2, neuroblastoma yields a similar level of GD3 but 10× higher levels of GD2 (44 and 31 mcg, respectively; refs. 9 and 10). GD2 and GD3 are also expressed on peripheral nerves and brain, and treatment with anti-GD2/GD3 MoAbs causes pain (11–13), but spares the central nervous system, presumably because of the blood–brain barrier.

Preclinical studies and clinical trials of vaccines in adults showed immunogenicity significantly augmented by (1) stabilizing GD2 and GD3 through lactone formation; (2)

**Authors' Affiliations:** Departments of [1]Pediatrics and [2]Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York

**Corresponding Author:** Brian H. Kushner, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 212-639-6793; Fax: 212-717-3239; E-mail: kushnerb@mskcc.org

**doi:** 10.1158/1078-0432.CCR-13-1012

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



## Translational Relevance

In preclinical models and clinical trials, immunotherapy using anti-GD2 or anti-GD3 monoclonal antibodies (MoAb) has proven beneficial against high-risk neuroblastoma (HR-NB) and melanoma. This article presents results of a phase I study of a vaccine designed to induce host anti-ganglioside antibodies that can replicate the antineoplastic activities of intravenously administered MoAbs. Patients also received β-glucan, which synergizes with anti-GD2/GD3 MoAbs. The vaccine/β-glucan treatment was well tolerated, and serological and minimal residual disease (MRD) responses were encouraging. Multiple factors may account for the excellent relapse-free survival despite prior relapse, which historically conferred a dismal prognosis. This phase I experience could have a major impact on treatment of HR-NB. Thus, a phase II trial is already in progress; the results might support vaccine/β-glucan as adjuvant after completion of the standard upfront HR-NB multimodality program. The latter now includes anti-GD2 MoAb but additional nontoxic therapy for MRD is eagerly sought, to help further improve outcome.

conjugating the resulting GD2L (L for lactone) and GD3L structures to the immunologic carrier protein keyhole limpet hemocyanin (KLH); and (3) combining GD2L-KLH and GD3L-KLH with the immunologic saponin adjuvant QS-21 (14–19). For both GD2L and GD3L, the lowest optimal dose was 30 mcg per vaccine (18,19). For QS-21, doses of 50 to 200 mcg were tested and found safe, with side effects of grade 3 erythema and flu-like symptoms lasting 2 to 4 days (20). When QS-21 became unavailable, we turned to OPT-821, which is a QS-21 equivalent based on $^{1}H$ and $^{13}C$ nuclear magnetic resonance, mass spectrometry, high-performance liquid chromatography studies, and overlapping immunogenicity and toxicity profiles in mice.

We now report a phase I trial involving children with HR-NB in second or later remission, treated with a GD2/GD3 vaccine containing OPT-821. Patients also received β-glucan, which has a broad range of immunostimulant effects (21–23) and synergizes with anti-GD2/GD3 MoAbs in preclinical studies (24,25). β-Glucans bind to CR3 (Mac-1), a complement receptor for iC3b, widely expressed on leukocytes (21). This receptor mediates the diapedesis of leukocytes through the endothelium and stimulates phagocytosis, degranulation, and tumor cytotoxicity. Many bacteria and fungi carry activating ligands for CR3, which renders them vulnerable to phagocytosis by neutrophils. Human tumors lack such molecules and CR3-mediated tumor lysis is suboptimal until it is activated by GM-CSF (26) or by ligands such as β-glucan (27).

## Materials and Methods

The primary objective of the Memorial Sloan-Kettering Cancer Center (MSKCC) protocol 05-075 phase I trial (Clinicaltrials.gov NCT00911560) was to find the maximally tolerated dose (MTD) in children of the immunologic adjuvant OPT-821 in a vaccine containing 2 neuroblastoma-associated antigens (GD2 and GD3). Secondary objectives were to obtain preliminary data on anti-GD2 and anti-GD3 immune response and anti-neuroblastoma activity against minimal residual disease (MRD).

### Patient selection

This study enrolled patients ≤21 years old with HR-NB who had previously relapsed but had achieved a ≥2nd complete/very good partial remission (CR/VGPR). Disease status was confirmed ≥3 weeks after other therapy by computed tomography, magnetic resonance imaging (MRI), $^{123}$I-metaiodobenzylguanidine (MIBG) scan, bone marrow histology (aspirates and biopsies from bilateral posterior and anterior iliac crests), and urine catecholamines. Using international criteria (28) expanded to include $^{123}$I-MIBG findings, complete remission was defined as absence of neuroblastoma by all studies, and VGPR was defined as the primary mass reduced ≥90%, no evidence of active distant disease, and urine catecholamines normal. There were no eligibility criteria/limits about prior therapy. Major organ toxicity was required to be ≤grade 3, except neurologic status had to be ≤grade 1, by the Common Terminology Criteria for Adverse Events, Version 3.0 (CTCAEv3.0), and the absolute lymphocyte and neutrophil counts each had to be ≥500/μL. Informed written consents for treatment and tests were obtained according to MSKCC Institutional Review Board rules.

### Study design

Treatment included 7 subcutaneous injections of the vaccine administered weeks 1–2–3–8–20–32–52 (Fig. 1). The vaccine contained 30 μg of GD2L and 30 μg of GD3L, each conjugated to KLH, for all patients, but OPT-821 dosing depended on dosage level and body-surface area. Four OPT-821 dosage levels were planned: 50, 75, 100, and 150 μg/m$^2$; the dose was "capped" with the maximal dose being 75, 110, 150, or 200 μg at the respective dosage levels.

Oral β-glucan was started week 6 (Fig. 1), to allow time for generation of antibodies. Dosage (40 mg/kg/day, 14 days on/14 days off) was identical for all patients and derived from the phase I MSKCC protocol 05-073 (Clinicaltrials.gov NCT00492167). β-Glucan [a botanical extract of sugar polymer consisting of (1 → 3),(1 → 6)-β-glycosidic linkage from baker's yeast, manufactured by Biotec Pharmacon ASA

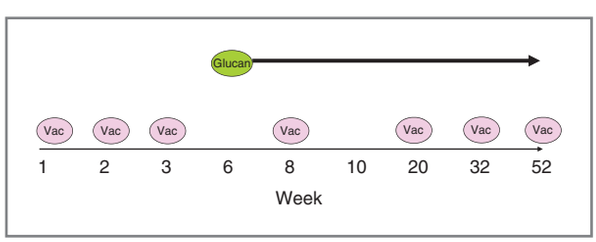


Figure 1. Protocol treatment schema. Glucan, β-glucan from yeast; Vac, GD2–GD3 vaccine containing OPT-821.

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



Inc.] was supplied as an aqueous solution at 20 mg/mL and stored at room temperature.

Patients continued on study in the absence of relapse (new lesion) and dose-limiting toxicity (DLT). Disease status was assessed every 10 to 12 weeks by extensive evaluations. Relapse-free survival (RFS) was calculated with the Kaplan–Meier method. Using the CTCAEv3.0, DLT was defined as: ≥grade 2 allergic reaction; ≥grade 2 autoimmune reaction; ≥grade 3 hematologic or non-hematologic toxicity; grade 3 injection site reaction; ≥grade 2 neurotoxicity in patients with no prior neurologic deficit, or worsening of a grade 1 neurotoxicity to grade 2. DLT assessment excluded toxicity related to disease activity, prior therapy, or cointerventions.

Vaccine injections were performed if liver function tests were ≤grade 1 toxicity. For grade 2 or 3 toxicity, injections were deferred until a return to ≤grade 1.

A standard dose-escalation schema was used: escalations were implemented if 0 of 3 or ≤1 of 6 patients at a given dosage level had DLT. At least 6 patients were studied at the MTD, or at the highest planned level (i.e., 150 mcg/m$^2$) if there were no DLTs.

### Materials

GD2 and GD3 were obtained from Matreya Inc., with GD2 prepared via extraction of GD1b from rabbit brain followed by treatment with bull testis β-galactosidase (19), and GD3 extracted from bovine buttermilk (18). GD2 and GD3 were >95% pure as determined by thin-layer chromatography and instant thin-layer chromatography (ITLC). Clinical grade KLH was acquired from Sigma Chemicals Inc., and clinical grade OPT-821 from Optimer Pharmaceuticals Inc.

For conjugation of gangliosides to KLH, ozone was used to cleave the ceramide double bond, an aldehyde group was introduced, and this product was coupled to amino groups on KLH by reductive amination as described (18). The ratio of antigen to KLH in the final conjugates was determined using the resorcinol method for measuring sialic acid and the Bio-Rad dye-binding method. The percent unconjugated antigen in the final vialed conjugates was determined by ITLC using MoAbs against each antigen; <10% unconjugated antigen was permitted. This vaccine was used under an IND held by MSKCC, BBIND 4976.

The GD2-KLH and GD3-KLH were converted to GD2L-KLH and GD3L-KLH by treatment with equal volume of conjugates and glacial acetic acid (v/v) × 3 to 4 hours at 37°C and lyophilized, as described (19). Vaccine was vialed in the MSKCC Clinical Grade Production Facility. Vials were stored at ≤−80°C. GD2L-KLH and GD3L-KLH were vialed together in sterile water containing 33 or 36 μg of each ganglioside per vial and lyophilized. OPT-821 was vialed in normal saline at 100 or 200 μg/0.5 mL, or at 150 μg/mL.

***Vaccine preparation.*** On the day of vaccination, the prescribed dose +10% of OPT-821 was diluted with normal saline to a final volume of 1.1 or 1.2 mL for the 33 or 36 μg vials of GD2L-KLH and GD3L-KLH, respectively. The resulting solution was refrigerated at 4°C ×1 hour. For administration, 1 mL (containing 30 μg of GD2L-KLH and of GD3L-KLH) was drawn into a syringe and injected subcutaneously in a single site.

***Serology.*** Anti-GD2 and anti-GD3 antibodies were detected by ELISA. In brief, 20 ng/well of GD2 or GD3 was coated on CDGH rows of 96-well microtiter plates and air-dried overnight. Blocking step was performed using 200 μL/well of 0.5% bovine serum albumin (BSA) in 1× PBS ×1 hour at room temperature, and washed with 1× PBS. Patient serum diluted in 1:10 and 1:30 in 0.5% BSA in duplicate was added to the designated wells with and without GD2/GD3 at 50 μL/well. For anti-GD2 assay, anti-GD2 antibody Hu3F8-IgG$_1$ was used to generate a standard curve at concentrations ranging from 1.23 to 100 ng/mL. Both standards and samples were incubated ×2.5 hours at 37°C. After washing with PBS, peroxidase-conjugated mouse anti-human IgG$_1$ (γ specific) at 1:1,000 dilution was added at 100 μL/well. Upon incubation at 4°C ×1 hour and washing with PBS, color reaction with chromogen *o*-phenylenediamine, and substrate hydrogen peroxide was added to the plates ×30 minutes at room temperature in the dark. Reaction was stopped using sulfuric acid, and optical density read using ELISA plate reader at 490 nm. For GD2 assay, test sera were quantified in ng/mL based on the standard curve; positivity was defined as an increase of at least 10 ng/mL over pretreatment levels. For GD3 assay, sera were defined as positive when titer at 1:10 was greater than 0.1 O.D. after background subtraction.

***MRD detection.*** Quantitative reverse transcription-PCR (qRT-PCR) was used as described (13,29,30) to assess MRD in heparinized bone marrow aspirates pooled from 4 sites (2–2.5 mL/site, from bilateral posterior and anterior iliac crests). The MRD marker panel included cyclin D1 (*CCND1*), GD2 synthase (*B4GALNT1*), ISL LIM homeobox 1 (*ISL1*), and paired-like homeobox 2b (*PHOX2B*). β2 microglobulin (β2*M*) was used as the endogenous control, and neuroblastoma cell line NMB7 as the positive control. Each sample was quantified using the comparative threshold cycle method as fold-difference relative to NMB7. All gene expression assays were from Applied Biosystems: *CCND1*: Hs00277039_m1; *B4GALNT1*: Hs00155195_m1; *ISL1*: Hs00158126_m1; *PHOX2B*: Hs00243679_m1; β2*M*: 4326319E. For each marker, positivity was defined as greater than the upper limit of normal. All samples were run in duplicates. MRD panel positivity was defined as any one of 4 markers being positive, and negativity as all 4 markers being negative.

## Results

### Patient characteristics

The study was completed with 15 patients (enrolled July 2009–November 2010) because there was no DLT and OPT-821 dosing reached that used in adults (Table 1). At enrollment, patients were 3.3 to 16.7 (median 8.1) years old. All patients had stage 4 neuroblastoma, 11 were in 2nd and 4 in ≥3rd CR/VGPR. Five (33%) patients had *MYCN*-amplified neuroblastoma. The time from diagnosis to first

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012

**Table 1.** Patients treated with bivalent vaccine and β-glucan

| Patient No. | Dose level[a] | *MYCN* amplified? | Time to first relapse | Sites of relapse | Treatment of relapse | Time from relapse to vaccine | MRD in BM pre/post | Serologic response[b] to: GD2 | Serologic response[b] to: GD3 | Outcome (time from first vaccine) |
|---|---|---|---|---|---|---|---|---|---|---|
| Patients in second CR | | | | | | | | | | |
| 1 | 1 | No | 11 mo | Tibia | CPT-TMZ(×12), RT, Thalidomide-Celecoxib | 26 mo | +/− | − | + (7) | RFS (48 mo) |
| 2 | 1 | No | 15 mo | Brain, abdomen | GTR, CPT, RT, CPT-TMZ(×2), IT-3F8, CIT, oral TMZ(×5), CRA, Ritux-Cyclo | 34 mo | +/n.d. | + (3) | + (7) | RFS (47 mo) |
| 3 | 2 | No | 17 mo | Abdomen | GTR, CPT-TMZ(×5), RT, Lenalidomide-Celecoxib | 7 mo | +/+ | + (4) | + (3) | RFS (46 mo) |
| 4 | 2 | No | 23 mo | Abdomen | GTR, CTV(×2), CIT, RT, 3F8, CRA | 25 mo | −/− | − | + (4) | RFS (45 mo) |
| 5 | 2 | No | 23 mo | Mandible, skull | ICE, RT, CPT-TMZ(×10), CRA | 23 mo | +/− | + (4) | + (7) | RFS (44 mo) |
| 6 | 4 | Yes | 59 mo | Pelvic nodes | GTR, CTV(×3), RT, CPT-TMZ, 3F8, CRA | 15 mo | −/− | − | + (6) | RFS (39 mo) |
| 7 | 4 | No | 22 mo | Thorax, bones | CTV(x2), GTR, CIT, CPT-TMZ(×9), RT, Ritux-Cyclo, 3F8, CRA | 40 mo | −/− | − | − | RFS (39 mo) |
| 8 | 4 | Yes | 30 mo | Femur, BM | CTV(×2), RT, CPT-TMZ(×5), CRA, ABT-751 (×3 yr) | 63 mo | +/+ | + (4) | + (6) | RFS (35 mo) |
| 9 | 4 | No | 8 mo | Paraspinal soft tissue, rib | CAV(×3), P/E(×2), GTR, CTV, RT, 3F8, CRA | 19 mo | +/− | − | − | RFS (33 mo) |
| 10 | 4 | No | 12 mo | Skull | CPT-TMZ(×7), RT, Ritux-Cyclo | 6 mo | +/+ | − | − | RFS (33 mo) |
| 11 | 4 | Yes | 26 mo | Sphenoid, BM | CTV(×2), ICE, RT, Bevacizumab-CPT-TMZ(×5) | 9 mo | +/− | + (4) | + (3) | Relapse (ischium; 2 mo). DoD (26 mo) |
| Patients in ≥3rd CR | | | | | | | | | | |
| 12 | 1 | Yes | 15 mo | Thorax | GTR, RT, CPT/TMZ(×5), 3F8, CRA, Thalidomide-Celecoxib | 15 mo | −/− | + (3) | + (7) | RFS (48 mo) |
| 13 | 3 | No | 47 mo | Thorax | Cyclo-Topo(×8), Lenalidomide-Celecoxib | 15 mo | +/− | + (2) | − | Relapse (BM, paravertebral; 5 mo); alive in CR (42 mo) |
| 14 | 3 | Yes | 21 mo | Tibia | CCV(×2), RT, Cyclo-Topo(×2), Ritux, Lenalidomide-Celecoxib | 10 mo | +/+ | + (4) | + (4) | RFS (40 mo) |
| 15 | 3 | No | 17 mo | R supra-clavicular | $^{131}$I-MIBG, RT | 3 mo | +/− | + (7) | − | Relapse (neck; 21 mo); alive in CR (42 mo) |

Abbreviations: BM, bone marrow; CAV, high-dose Cyclo-doxorubicin-vincristine; CCV, high-dose Cyclo-CPT-vincristine (7); CIT, high-dose carboplatin-CPT-TMZ (6); CPT, irinotecan; CRA, 13-*cis*-retinoic acid; CTV, high-dose Cyclo-Topo-vincristine (5); Cyclo, cyclophosphamide; DoD, died of disease; GTR, gross total resection; ICE, high-dose ifosfamide-carboplatin-etoposide (8); IT-3F8, intrathecal $^{131}$I-3F8 (4); MRD, minimal residual disease; n.d., not done; P/E, cisplatin-etoposide; RFS, relapse-free survival; Ritux, rituximab; RT, local radiotherapy; TMZ, temozolomide; Topo, topotecan.

[a]OPT-821 dose level 1 = 50 μg/m$^2$, dose level 2 = 75 μg/m$^2$, dose level 3 = 100 μg/m$^2$, and dose level 4 = 150 μg/m$^2$.

[b]Number of prior vaccine injections on this protocol is in parentheses.

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



relapse was <12 months ($n = 2$), 12 to 18 months ($n = 5$), 19 to 24 months ($n = 4$), and >24 months ($n = 4$). Retrieval therapy before study enrollment included local control with radiotherapy alone ($n = 7$), radiotherapy plus surgery ($n = 7$), strongly myelosuppressive chemotherapy ($n = 8$),[5–8] and nonimmunosuppressive treatments such as irinotecan-temozolomide ($n = 9$; refs. 3 and 4)[3,4] and anti-GD2 MoAb (13; $n = 5$); no patient underwent stem-cell transplantation as part of retrieval. These retrieval treatments were for relapses that were localized in 10 patients, including soft tissue ($n = 7$) or osteomedullary by MIBG and MRI ($n = 3$), and widespread in 5 patients, including bone marrow by histology and MIBG scan ($n = 2$), multifocal MIBG osteomedullary with ($n = 1$) or without ($n = 1$) soft tissue, and retroperitoneal nodes plus brain ($n = 1$).

### Survival and toxicity

Thirteen of fifteen patients received all 7 protocol-prescribed injections of vaccine, including 12 patients who remain relapse-free at 21+ to 36+ (median 29+) months and one who had a focal relapse (supraclavicular node) at 21 months. Two patients had early focal relapses (2.3 and 4.6 months). All 3 relapses were among patients receiving 100 to 150 μg/m$^2$ of OPT-821, including 1 with *MYCN*-amplified and 2 with *MYCN*-nonamplified neuroblastoma. RFS was 87% ± 9% at 12 and 80% ± 10% at 24 months (Fig. 2). Overall survival was 93% ± 6% (follow-up >25 months). One relapse-free survivor received a cycle of chemotherapy because of radiologic findings deemed suspicious for soft tissue relapse of his *MYCN*-amplified neuroblastoma; laparotomy revealed no evidence of neuroblastoma, so patient was allowed to resume the vaccine/β-glucan immunotherapy.

Treatment was well tolerated, with no DLT, delays in administration of vaccine, co-interventions, or neurologic sequelae. There was no relation between OPT-821 dosage and side effects. Injections were painful and elicited grade 1 local reactions in 12 patients (varying from 1 to 4 injections per patient) and a grade 2 local reaction in one patient (dose level 1, with injection #7); these side effects subsided within 24 hours. No other grade 2 toxicities of protocol treatment were noted. One patient had a grade 3 elevation in liver function enzymes on day 5 after injection #2 (dose level 1). The only other grade 3 to 4 toxicities—all self-limited and attributable to prior therapy or intercurrent events—were concurrent hypocalcemia and hypokalemia (1 patient), lymphopenia (1 patient), and neutropenia (1 patient).

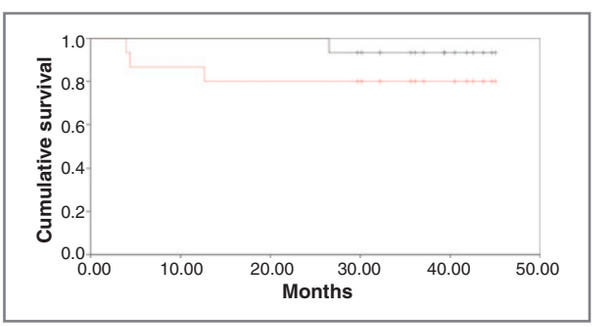


Figure 2. Relapse-free survival (red) and overall survival (black) of the 15 study patients from the start of the vaccine.

### Serological response

Patient serum for assessment of anti-GD2 and anti-GD3 titers was collected before vaccine injection #1 and after the subsequent injections. Seropositive response was defined as negative titer at baseline, and positive titer anytime, whereas seronegative response was defined as negative titer at baseline and all subsequent time points tested. Three of fifteen patients had seronegative response. The rest of them became seropositive against GD2 alone ($n = 2$), GD3 alone ($n = 3$), or both ($n = 7$; Table 1). Anti-GD2 seropositivity was documented after cycles #2 ($n = 1$), #3 ($n = 2$), #4 ($n = 5$), and #7 ($n = 1$). Anti-GD3 seropositivity was documented after cycles #3 ($n = 2$), #4 ($n = 2$), #6 ($n = 2$), and #7 ($n = 4$) (Fig. 3). Seronegativity did not correlate with relapse.

### MRD response

At study enrollment, all 15 patients had complete remission in bone marrow by histology. Response with respect to MRD was assessed by comparing bone marrow collected at baseline before vaccine injection #1 versus after the last injection. Of 11 patients who were MRD positive at baseline, 6 became MRD negative, 4 remained MRD positive, and 1 patient in continual remission was not retested for MRD because there was no available sample (Table 1). Four patients were MRD negative at baseline and remained MRD negative. MRD nonresponse did not correlate with relapse.

## Discussion

This phase I trial in patients with HR-NB in ≥2nd CR/VGPR assessed the toxicity of escalating dosages of the immunologic adjuvant OPT-821 in a bivalent vaccine containing GD2L and GD3L, each covalently attached to the immunologic carrier protein KLH. The patients also took the oral immunostimulant β-glucan. Because thirteen of fifteen study patients received all 7 injections of the protocol, the study's primary objective about safe dosing of OPT-821 was achieved. Treatments were devoid of acute toxicities aside from local reactions immediately postinjection. No delayed toxicities developed, alleviating concerns about neurotoxicity given GD2/GD3 expression on peripheral nerves and in brain. Prolonged oral intake of β-glucan by the children in this study was uncomplicated, consistent with the absence of major side effects in preclinical animal models and in human adults.

Although the absence of toxicity with the bivalent vaccine and β-glucan seems well established, anti-neuroblastoma efficacy should be interpreted with great caution. The study patients achieved excellent RFS (Fig. 2), in contrast to dismal predictions based on the international experience with relapsed HR-NB (31–33), and without myeloablative therapy with autologous stem-cell transplantation, which is widely used as consolidation of first or subsequent remissions. Yet the contributory role of, for example, β-glucan to

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



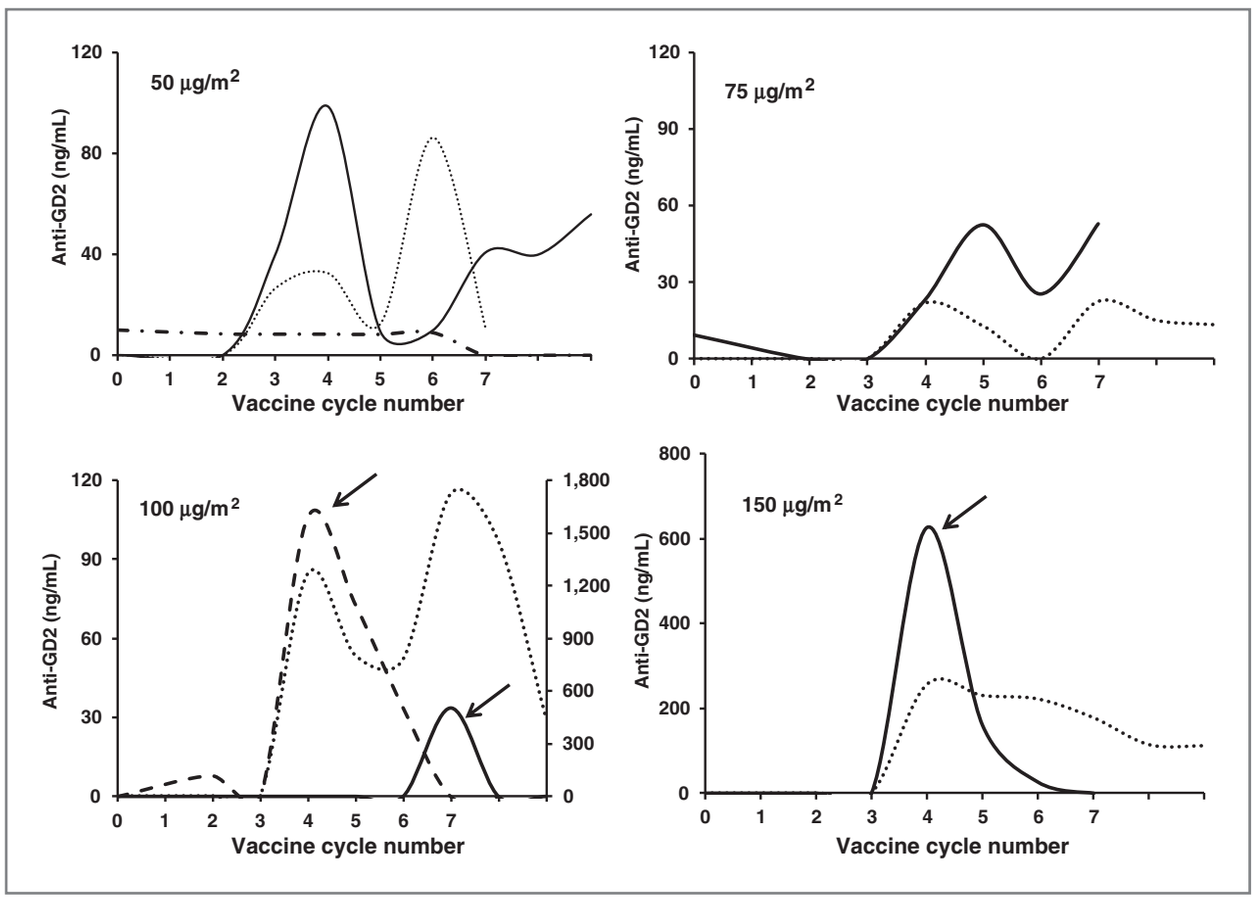


Figure 3. Antibody response against GD2 in ng/mL (*y*-axis) after each vaccine cycle number (*x*-axis) for individual patients who turned seropositive, grouped according to the dose level of OPT-821 (50, 75, 100, and 150 μg/m$^2$). At 100 μg/m$^2$ dose level, one patient (dash) had unusually high antibody titer plotted on the secondary *y*-axis. Patients who did not mount any anti-GD2 antibody response were not included in these plots (1/3 patients at 75 μg/m$^2$ dose level and 4/6 patients at 150 μg/m$^2$ dose level). The 3 patients who progressed were marked with black arrows.

RFS is uncertain, and major confounding factors included patient selection and the proximity and complexity of therapy before vaccine treatment. Nevertheless, twelve of fifteen patients mounted an anti-disialoganglioside immune response, and bone marrow MRD response (from positive to negative) occurred in 60% of evaluable patients. All 3 patients who progressed had complete remission of bone marrow MRD. It is of note that 2 patients had isolated progression (1 in a neck node and 1 in left ichium) with continual marrow remission for 17 months and 24+ months. The third patient progressed with a new paraspinal soft tissue mass with only 1 of 6 marrow samples turning positive, and had negative marrow aspirates and biopsies (total of 60 samples) alive and well at 41+ months since. The inability of bone marrow MRD to detect extramedullary isolated relapse was analyzed in a recent publication, showing early negative MRD in bone marrow after immunotherapy was less predictive of late or nonmarrow relapse among patients with high-risk stage 4 neuroblastoma (34).

Vaccine therapy in neuroblastoma has previously focused on T-cell immunity. Syngeneic or allogeneic neuroblastoma transfected with interleukin-2 can stimulate anti-neuroblastoma IgG (35). Allogeneic neuroblastoma transfected with both interleukin-2 and lymphotactin has induced major responses in patients (36,37). Dendritic cells expanded *in vitro* and "pulsed" with crude cell lysates or tumor RNA have also been tested in children (38,39). Unfortunately, to date, specific CTLs (e.g., against survivin) have not been shown to home to neuroblastoma in patients (40). Furthermore, T-cell immunity has been limited by the state of immunosuppression following dose-intensive chemotherapy. In this study, the successful induction of anti-GD2 and anti-GD3 antibodies using lactone-KLH in the presence of OPT-821 in patients despite prior history of immunosuppressive chemotherapy is encouraging.

In preclinical models (41,42) and clinical trials (11–13), immunotherapy using anti-GD2 or anti-GD3 MoAbs has shown activity against neuroblastoma and melanoma. Benefit is optimal in the adjuvant setting, with ablation of micrometastasis (43). The antineoplastic mechanisms include opsonization, antibody-dependent cellular cytotoxicity (ADCC), and complement-mediated cytotoxicity (CMC). The ADCC involves leukocytes of all types, including neutrophils, macrophages, and natural killer cells. Our

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



bivalent vaccine is constructed with the purpose of inducing host anti-ganglioside antibodies that can replicate the antineoplastic effects of exogenously administered MoAbs.

Various strategies have undergone clinical testing to induce or augment immune-mediated attack against cancer (44). Few clinical trials, however, have used humoral vaccines against solid tumors in children, especially with ADCC and CMC as the principal underlying immunocytotoxic mechanisms (45). Also, an antineoplastic role for myeloid cells (e.g., neutrophils) has received scant attention (27). A treatment program combining an anti-neuroblastoma vaccine with an agent that enhances granulocyte cytotoxicity (i.e., β-glucan) represents a novel strategy to maximize this myeloid effect in the emerging field of immunotherapy.

The setting of this protocol is an ideal model system to assess anticancer vaccine therapy: patients with a low disease burden, but at great risk for relapse; target antigens highly expressed on the cancerous but not normal tissues; immunological adjuvants (KLH and OPT-821) to optimize induction of antibodies against the target antigens (GD2 and GD3); and a nontoxic oral agent (β-glucan) to enhance effector cytotoxicity. The basis for emphasis on multivalent vaccines is tumor-cell heterogeneity, variability of the immune response among patients, and the correlation between the concentration of tumor-selective antibody and effector mechanisms (opsonization, ADCC, and CMC). In the adjuvant setting, prolonged presence of antineoplastic antibodies should be more efficient against MRD. Maintenance of antibodies over months or years is more readily achieved with vaccines than MoAbs where repeated administrations are necessary and human anti-mouse or anti-human blocking antibodies can develop. We speculate that the fluctuations of anti-GD2 titer in vaccinated patients could represent multiple idiotypes within an idiotypic network whereas unimodal anti-GD2 response could represent ineffective idiotypes associated with an unfavorable outcome (46–48). Alternatively, these fluctuations could represent random oscillations of unknown significance.

In conclusion, the bivalent-vaccine/β-glucan immunotherapy program engenders no major toxicity, even in heavily prior-treated children, and is readily transportable to multiple institutions. Patients have no neuropathic pain, unlike patients receiving intravenous anti-GD2 or anti-GD3 MoAbs. Serological and MRD responses were encouraging. Multiple factors may account for the excellent RFS. This limited experience provides support for pursuing an aggressive curative strategy against relapsed HR-NB. In addition, unlike most pediatric phase I studies, this one could have a major impact on treatment of HR-NB. Thus, a phase II trial is already in progress, using the phase I study's maximum OPT-821 dosage (150 $\mu g/m^2$); the results might support vaccine-glucan as adjuvant after completion of the standard upfront HR-NB multimodality program. The latter now includes anti-GD2 MoAb (12) but additional nontoxic therapy for MRD is eagerly sought, to help further improve outcome.

## Disclosure of Potential Conflicts of Interest

G. Ragupathi has ownership interest (including patents) in MabVax Therapeutics. G. Ragupathi is a consultant/advisory board member of MabVax Therapeutics. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** B.H. Kushner, S. Modak, K. Kramer, N.-K.V. Cheung

**Development of methodology:** B.H. Kushner, I.Y. Cheung, K. Kramer, G. Ragupathi, N.-K.V. Cheung

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** B.H. Kushner, I.Y. Cheung, K. Kramer, G. Ragupathi, N.-K.V. Cheung

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** B.H. Kushner, I.Y. Cheung, S. Modak, K. Kramer, N.-K.V. Cheung

**Writing, review, and/or revision of the manuscript:** B.H. Kushner, I.Y. Cheung, S. Modak, K. Kramer, N.-K.V. Cheung

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** B. H. Kushner, N.-K.V. Cheung

**Study supervision:** B.H. Kushner, G. Ragupathi

## Grant Support

This work is supported in part by grants from the National Institutes of Health (CA10450), Bethesda, MD; the Thrasher Foundation, Salt Lake City, UT; the Gerber Foundation, Fremont, MI; the Robert Steel Foundation, New York, NY; and Katie's Find A Cure Fund, New York, NY.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 12, 2013; revised November 11, 2013; accepted November 27, 2013; published OnlineFirst February 11, 2014.

## References

1. Maris JM. Recent advances in neuroblastoma. N Engl J Med 2010;362:2202–11.
2. Kushner BH, Kramer K, Modak S, Cheung NK. Sensitivity of surveillance studies for detecting asymptomatic and unsuspected relapse of high-risk neuroblastoma. J Clin Oncol 2009;27:1041–6.
3. Kushner BH, Kramer K, Modak S, Cheung NK. Irinotecan plus temozolomide for relapsed or refractory neuroblastoma. J Clin Oncol 2006;24:5271–6.
4. Kramer K, Kushner BH, Modak S, Pandit-Taskar N, Smith-Jones P, Zanzonico P, et al. Compartmental intrathecal radioimmunotherapy: results for treatment for metastatic CNS neuroblastoma. J Neurooncol 2010;97:409–18.
5. Kushner BH, Kramer K, Modak S, Qin LX, Cheung NK. Differential impact of high-dose cyclophosphamide, topotecan, and vincristine in clinical subsets of patients with chemoresistant neuroblastoma. Cancer 2010;116:3054–60.
6. Kushner BH, Kramer K, Modak S, Cheung NK. High-dose carboplatin-irinotecan-temozolomide: treatment option for neuroblastoma resistant to topotecan. Pediatr Blood Cancer 2011;56:403–8.
7. Kushner BH, Kramer K, Modak S, Yataghene K, Cheung NK. High-dose cyclophosphamide-irinotecan-vincristine for primary refractory neuroblastoma. Eur J Cancer 2011;47:84–9.
8. Kushner BH, Modak S, Kramer K, Basu EM, Roberts SS, Cheung NK. Ifosfamide, carboplatin, and etoposide for neuroblastoma: a high-dose salvage regimen and review of the literature. Cancer 2013;119:665–71.
9. Hamilton WB, Helling F, Lloyd KO, Livingston PO. Ganglioside expression on human malignant melanoma assessed by quantitative immune thin-layer chromatography. Int J Cancer 1993;53:566–73.
10. Zhang S, Cordon-Cardo C, Zhang HS, Reuter VE, Adluri S, Hamilton WB, et al. Selection of tumor antigens as targets for immune attack

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



using immunohistochemistry. I. Focus on gangliosides. Int J Cancer 1997;73:42–9.
11. Houghton AN, Mintzer D, Cordon-Cardo C, Welt S, Fliegel B, Vadhan S, et al. Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. Proc Natl Acad Sci U S A 1985;82:1242–6.
12. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 2010;363:1324–34.
13. Cheung NK, Cheung IY, Kushner BH, Ostrovnaya I, Chamberlain E, Kramer K, et al. Murine anti-GD2 monoclonal antibody 3F8 combined with granulocyte-macrophage colony-stimulating factor and 13-cis-retinoic acid in high-risk patients with stage 4 neuroblastoma in first remission. J Clin Oncol 2012;30:3264–70.
14. Ritter G, Boosfeld E, Calves MJ, Oettgen HF, Old LJ, Livingston PO. Antibody response to immunization with purified GD3 ganglioside and GD3 derivatives (lactones, amide and gangliosidol) in the mouse. Immunobiology 1990;182:32–43.
15. Ritter G, Boosfeld E, Adluri R, Calves M, Oettgen HF, Old LJ, et al. Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide and gangliosidol) in patients with malignant melanoma. Int J Cancer 1991;48:379–85.
16. Kensil CR, Patel U, Lennick M, Marciani D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 1991;146:431–7.
17. Helling F, Shang A, Calves M, Zhang S, Ren S, Yu RK, et al. GD3 vaccines for melanoma: superior immunogenicity of keyhole limpet hemocyanin conjugate vaccines. Cancer Res 1994;54:197–203.
18. Ragupathi G, Meyers M, Adluri S, Howard L, Musselli C, Livingston PO. Induction of antibodies against GD3 ganglioside in melanoma patients by vaccination with GD3-lactone-KLH conjugate plus immunological adjuvant QS-21. Int J Cancer 2000;85:659–66.
19. Ragupathi G, Livingston PO, Hood C, Gathuru J, Krown SE, Chapman PB, et al. Consistent antibody response against ganglioside GD2 induced in patients with melanoma by a GD2 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant QS-21. Clin Cancer Res 2003;9:5214–20.
20. Livingston PO, Adluri S, Helling F, Yao TJ, Kensil CR, Newman MJ, et al. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 1994;12:1275–80.
21. Yan J, Vetvicka V, Xia Y, Coxon A, Carroll MC, Mayadas TN, et al. Beta-glucan, a "specific" biologic response modifier that uses antibodies to target tumors for cytotoxic recognition by leukocyte complement receptor type 3 (CD11b/CD18). J Immunol 1999;163:3045–52.
22. Taylor PR, Brown GD, Reid DM, Willment JA, Martinez-Pomares L, Gordon S, et al. The $\beta$-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. J Immunol 2002;169:3876–82.
23. Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, et al. Mechanism by which orally administered $\beta$(1,3)-glucans function with anti-tumor monoclonal antibodies to mediate tumor regression and tumor-free survival. J Immunol 2004;173:797–806.
24. Cheung NK, Modak S. Oral (1 $\rightarrow$ 3),(1 $\rightarrow$ 4)-$\beta$-D-glucan synergizes with antiganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res 2002;8:1217–23.
25. Cheung NK, Modak S, Vickers A, Knuckles B. Orally administered beta-glucans enhance anti-tumor effects of monoclonal antibodies. Cancer Immunol Immunother 2002;51:557–64.
26. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993;11:1466–77.
27. Cheung IY, Hsu K, Cheung NK. Activation of peripheral-blood granulocytes is strongly correlated with patient outcome after immunotherapy with anti-GD2 monoclonal antibody and granulocyte-macrophage colony-stimulating factor. J Clin Oncol 2012;30:426–432.
28. Ross GD. Role of the lectin domain of Mac-1/CR3 (CD11b/CD18) in regulating intercellular adhesion. Immunol Res 2002;25:219–27.
29. Cheung IY, Lo Piccolo MS, Kushner BH, Cheung NK. Early molecular response of marrow disease to biologic therapy is highly prognostic in neuroblastoma. J Clin Oncol 2003;21:3853–8.
30. Cheung IY, Feng Y, Gerald W, Cheung NK. Exploiting gene expression profiling to identify novel minimal residual disease markers of neuroblastoma. Clin Cancer Res 2008;14:7020–7.
31. London WB, Castel V, Monclair T, Ambros PF, Pearson AD, Cohn SL, et al. Clinical and biologic features predictive of survival after relapse of neuroblastoma: a report from the International Neuroblastoma Risk Group project. J Clin Oncol 2011;29:3286–92.
32. Simon T, Berthold F, Borkhardt A, Kremens B, De Carolis B, Hero B. Treatment and outcomes of patients with relapsed, high-risk neuroblastoma: results of German trials. Pediatr Blood Cancer 2011;56:578–83.
33. Cole KA, Maris JM. New strategies in refractory and recurrent neuroblastoma: translational opportunities to impact patient outcome. Clin Cancer Res 2012;18:2423–8.
34. Cheung IY, Feng Y, Cheung NK. Early negative minimal residual disease in bone marrow after immunotherapy is less predictive of late or non-marrow relapse among patients with high-risk stage 4 neuroblastoma. Pediatr Blood Cancer 2013;60:E32–E34.
35. Russell HV, Strother D, Mei Z, Rill D, Popek E, Biagi E, et al. A phase 1/2 study of autologous neuroblastoma tumor cells genetically modified to secrete IL-2 in patients with high-risk neuroblastoma. J Immunother 2008;31:812–9.
36. Rousseau RF, Haight AE, Hirschmann-Jax C, Yvon ES, Rill DR, Mei Z, et al. Local and systemic effects of an allogeneic tumor cell vaccine combining transgenic human lymphotactin with interleukin-2 in patients with advanced or refractory neuroblastoma. Blood 2003;101:1718–26.
37. Louis CU, Brenner MK. Cellular immunotherapy for neuroblastoma: a review of current vaccine and adoptive T cell therapeutics. Curr Pharm Des 2009;15:424–9.
38. Geiger JD, Hutchinson RJ, Hohenkirk LF, McKenna EA, Yanik GA, Levine JE, et al. Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. Cancer Res 2001;61:8513–9.
39. Caruso DA, Orme LM, Amor GM, Neale AM, Radcliff FJ, Downie P, et al. Results of a phase I study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children with Stage 4 neuroblastoma. Cancer 2005;103:1280–91.
40. Coughlin CM, Fleming MD, Carroll RG, Pawel BR, Hogarty MD, Shan X, et al. Immunosurveillance and survivin-specific T-cell immunity in children with high-risk neuroblastoma. J Clin Oncol 2006;24:5725–34.
41. Nasi ML, Meyers M, Livingston PO, Houghton AN, Chapman PB. Anti-melanoma effects of R24, a monoclonal antibody against GD3 ganglioside. Melanoma Res 1997;7:S155–62.
42. Zhang H, Zhang S, Cheung NK, Ragupathi G, Livingston PO. Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases. Cancer Res 1998;58:2844–9.
43. Kim SK, Wu X, Ragupathi G, Gathuru J, Koide F, Cheung NK, et al. Impact of minimal tumor burden on antibody response to vaccination. Cancer Immunol Immunother 2011;60:621–7.
44. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature 2011;480:480–9.
45. Navid F, Armstrong M, Barfield RC. Immune therapies for neuroblastoma. Cancer Biol Ther 2009;8:874–82.
46. Jerne NK. The generative grammar of the immune system. EMBO 1985;4847–52.
47. Raychaudhuri S, Saeki Y, Chen JJ, Kohler H. Analysis of the idiotypic network in tumor immunity. J Immunol 1987;139:3902–10.
48. Cheung NK, Guo HF, Heller G, Cheung IY. Induction of Ab3 and Ab3′ antibody was associated with long-term survival after anti-GD2 antibody therapy of stage 4 neuroblastoma. Clin Cancer Res 2000;6:2653–60.

Published OnlineFirst February 11, 2014; DOI: 10.1158/1078-0432.CCR-13-1012



Clinical Cancer Research

# Phase I Trial of a Bivalent Gangliosides Vaccine in Combination with β-Glucan for High-Risk Neuroblastoma in Second or Later Remission

Brian H. Kushner, Irene Y. Cheung, Shakeel Modak, et al.

*Clin Cancer Res* 2014;20:1375-1382. Published OnlineFirst February 11, 2014.