Immunomodulatory Monoclonal Antibodies Combined with Peptide Vaccination Provide Potent Immunotherapy in an Aggressive Murine Neuroblastoma Model

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Abstract

Purpose: Neuroblastoma is one of the commonest extracranial tumors of childhood. The majority of patients present with metastatic disease for which outcome remains poor. Immunotherapy is an attractive therapeutic approach for this disease, and a number of neuroblastoma tumor antigens have been identified. Here, we examine the therapeutic potential of combining immunomodulatory monoclonal antibodies (mAb) with peptide vaccination in murine neuroblastoma models.

Experimental Design: Neuroblastoma-bearing mice were treated with mAb targeting 4-1BB, CD40, and CTLA-4 alone, or in combination with a peptide derived from the tumor antigen survivin (GWEDPPNDI). Survivin-specific immune response and therapeutic efficacy were assessed.

Results: In the Neuro2a model, treatment of established tumor with anti-4-1BB, anti-CD40, or anti-CTLA-4 mAb results in tumor regression and long-term survival in 40% to 60% of mice. This is dependent on natural killer (NK) and CD8⁺ T cells and is associated with tumor CD8⁺ lymphocyte infiltrate. Successful therapy is achieved only if mAb is given to mice once tumors are established, suggesting dependence on sufficient tumor to provide antigen. In the more aggressive AgN2a and NXS2 models, single-agent mAb therapy provides ineffective therapy. However, if mAb (anti-CTLA-4) is given in conjunction with survivin peptide vaccination, then 60% long-term survival is achieved. This is associated with the generation of survivin-specific T-cell immunity, which again is only shown in the presence of tumor antigen.

Conclusions: These data suggest that the combination of antigen and costimulatory mAb may provide effective immunotherapy against neuroblastoma and may be of particular use in the minimal residual disease setting.

Introduction

Neuroblastoma, an embryonal tumor originating from sympathetic neural crest cells, is one of the commonest extracranial malignancies of childhood, accounting for 6% to 8% of all childhood cancers and more than 15% of pediatric cancer deaths. More than 50% of children have metastatic disease at presentation, and long-term survival is seen in only around 30% to 40% of these patients despite intensive multimodal therapies (1, 2). Treatment-associated mortality is significant (5%–8%), so there is little room to further intensify therapies (3).

Immunotherapy is an attractive alternative therapeutic option for these children as it potentially offers a more specific and less toxic treatment than conventional therapies.

Endogenous cellular and humoral antitumor immune responses in children with neuroblastoma are well established, and it is the most frequent human tumor to spontaneously undergo regression (4). Lymphoid infiltration is found in approximately 25% of tumors, and has been correlated favorably with outcome (5, 6). A number of tumor antigens have been identified including survivin, tyrosine hydroxylase, MYC-N, GD2, and a number of cancer testis antigens and endogenous immune responses to some of these have been identified (4). Coughlin and colleagues (2006) showed survivin-specific CD8⁺ T cells in the peripheral blood of 8 out of 9 children with high-risk neuroblastoma, not seen in healthy controls, accounting for up to 0.64% of the circulating CD8⁺ lymphocytes (7). Although tumor growth was not controlled in vivo, the majority of these T cells showed cytotoxicity against human neuroblastoma following in vitro restimulation. Such T cells may be inadequate to eradicate the tumors in patients due to limited tumor antigen, lack of costimulation, and/or

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Translational Relevance
The majority of children with neuroblastoma present with metastatic disease for which long-term survival remains poor, despite intensive multimodal therapies. Immunotherapy is an attractive therapeutic approach for these children, potentially being more specific and less toxic than conventional therapies. Survivin is, particularly, a promising immunotherapy target as it is expressed in 80% to 100% of high-risk tumors and only minimally in normal tissue. Spontaneous anti-survivin T-cell responses have also been reported in children with neuroblastoma. Immunomodulatory monoclonal antibodies targeting costimulatory molecules (CD40, 4-1BB, OX40, PD-1) have entered clinical trial in syngeneic tumor models (12–15).

Materials and Methods
Animals and cells
A/J mice were supplied by Harlan. Animal experiments were cleared through local ethical committee and carried out under Home Office licenses PPL30/2450 and 30/2451. Neuro2a (European Collection of Animal Cell Cultures), AgN2a (Dr. Rimas Orentas, Medical College of Wisconsin, Milwaukee, WI), and NXS2 (Dr. Holger Lode, Charité Children’s Hospital, Berlin, Germany) cell lines were maintained in Dulbecco’s modified Eagle medium supplemented with 2 mmol/L glutamine, 0.1 mmol/L pyruvate, 100 IU/mL penicillin and streptomycin, 50 μmol/L β-mercaptoethanol (AgN2a). Splenocytes were maintained in RPMI containing 2 mmol/L glutamine, 1 mmol/L pyruvate, 100 IU/mL penicillin and streptomycin, 50 μmol/L β-mercaptoethanol, and 10% FCS (Invitrogen).

Antibodies
Hybridomas for LOB12.3 (anti-4-1BB) and Mc106A5 (anti-BCL1 Id, irrelevant control) were generated in house (20, 21). The 3/23 (anti-CD40) hybridoma was originally provided by G. Klaus, National Institute for Medical Research, London (22). The UC10-4F10-11 (anti-CTLA-4) hybridoma was obtained from the American Type Culture Collection. Antibodies for flow cytometry were obtained from BD Biosciences unless otherwise stated.

Murine neuroblastoma therapy models
Age-matched 8- to 12-week-old A/J mice were injected subcutaneously with 2 × 10^6 freshly prepared tumor cells (> 90% viability) in 100 μL PBS on day 0 and received antibody/peptide vaccine as specified in individual experiments. All antibodies were given by intraperitoneal injection diluted in PBS. Survivin (GWEPDDNPI) and control (SINFEKL or FEANGNLI) peptides in PBS were emulsified in equal volumes of incomplete Freund’s adjuvant (IFA) before intradermal injection. Tumor diameter was measured regularly and mice culled when cross-sectional area exceeded 225 mm^2.

Where indicated CD8^+/-CD4^+/-natural killer (NK) cells were removed by administration of depleting antibodies as previously described (23). Five hundred micromolars anti-CD8 mAb (YTS169), 1 mg anti-CD4 mAb (YTA 3.1.2), or 200 μL of a 1 in 10 dilution of anti-asialo GM1 (polyclonal mAbs targeting a number of these receptors (CTLA-4, CD40, 4-1BB, PD-1) have entered clinical trial in adult oncology patients, showing considerable promise. Ipilimumab, a human anti-CTLA-4 mAb, has been recently granted U.S. Food and Drug Administration approval for first-line treatment of metastatic melanoma, having been shown to offer survival advantage in phase III trial in this population (16). Smaller, early-phase studies of anti-CTLA-4 mAbs have suggested potential benefit in a number of other adult malignancies (17, 18). Although earlier in clinical development, a number of other agents (e.g., anti-CD40, anti-PD-1 and anti-4-1BB) are also showing promise in adult oncology patients (19). However, despite these encouraging results, there is, as yet, no reported pediatric experience of this class of agents.

Here, we show that immunostimulatory mAbs, either alone or in combination with peptide vaccine, can be used to generate potent antitumor immunity in murine neuroblastoma models.
rabbit anti-asialo GM1, Wako Chemicals) were injected intraperitoneally every 4 to 5 days.

**In vitro proliferation and viability assays**

During culture, 0.5 μCi [³H] thymidine per well was added for the last 16 hours, after which cells were harvested onto glass fibers with an automated harvester. [³H] thymidine incorporation was subsequently determined via liquid scintillation counting.

Cells were assessed for viability by flow cytometry after staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (AnV/PI) as detailed previously (24).

**Immunofluorescence**

OCT (RA Lamb) frozen tumor sections were fixed in 100% acetone for 10 minutes at 4°C, and nonspecific binding was blocked by 30 minutes preincubation with PBS containing 5% normal goat serum and 2% bovine serum albumin (BSA; pH 7.4). Sections were incubated with rat anti-mouse CD8α, CD4 (both BD Pharmingen), or NK2A/C/E (eBiosciences) diluted in 2% BSA/PBS, followed by Alexa Fluor 488-conjugated goat anti-rat immunoglobulin G (Life Technologies). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (Sigma) and sections mounted in Vectashield (Vector Laboratories). Images were collected using a CKX41 inverted microscope reflected fluorescence system equipped with a CC12 color camera running under Cell B software (Olympus), using a Plan Achromat 10X/0.25 objective lens (Olympus).

**Flow cytometry**

Flow cytometry was conducted as described previously (25) with samples assessed on a FACSCanto II and data analyzed using FACSDiva software (all BD Biosciences). To determine surface expression of 4-1BB, CD40, or CTLA-4, cells were labeled with 10 μg/mL unlabeled mAb (in-house) before incubation with R-phycoerythrin-conjugated anti-CD45 (BD Pharmingen) or APC-labeled anti-mouse CD3ε (clone 145-2c11).

**Intracellular IFN-γ**

Mice were immunized with mAb and/or peptide as specified. Splenocytes were cultured for 6 days with control (FEANGNL1) or GWEPPDNP1-pulsed, irradiated splenocytes in the presence of 20 IU/mL rHu IL-2 for 6 days. A standard chromium-51 (⁵¹Cr) release assay was then used to assess cytotoxic activity of splenic effectors, as previously described (13). ⁵¹Cr-labeled target cells were incubated with splenocytes for 5 hours under tissue culture conditions (5% CO₂, 37°C), centrifuged at 500 × g for 5 minutes before estimation of ⁵¹Cr release from supernatant using a gamma counter (Wallace 1470 WIZARD, PerkinElmer). Maximum release of radioactivity was calculated from target cells using 150 μL of 1% Nonidet P-40. Percentage specific release was calculated as (sample release – spontaneous release)/(maximum release – spontaneous release) × 100.

**Adoptive transfer assay**

Splenocytes from naïve A/J mice were pulsed with 25 μg/mL GWEPPDNP1 or control (FEANGNL1) peptide for 1 hour at 37°C, washed once with FCS-free RPMI, and then stained with high (5 μmol/L) and low (0.5 μmol/L) concentrations of carboxyfluorescein succinimidyl ester (CFSE), respectively. A 1:1 ratio of target (T; GWEPPDNP1 pulsed) to nontarget (NT; FEANGNL1 pulsed) cells were injected intravenously into recipient mice. After 24 hours, mice were culled and the splenic T:NT ratio remaining assessed by flow cytometry (FACSCanto II).

**Statistical analysis**

All statistical analysis was conducted using GraphPad Prism version 5 for Windows (GraphPad software). The statistical significance between treatment groups in the tumor models was analyzed using the log-rank test comparing survival curves. The statistical significance between IFN-γ responses in different groups of treated mice was calculated using Student t test. The statistical significance between CTL killing was compared using a one-way ANOVA.

**Results**

**Anti-4-1BB treatment of established, weakly immunogenic tumors resulted in resolution of tumor, long-term survival, and protection from tumor rechallenge**

Initially, therapeutic activity of the immunomodulatory mAbs was explored in the Neuro2a neuroblastoma model. This cell line expresses relatively low levels of MHC class I
but has been shown to be susceptible to T-cell–mediated immunotherapies (26). Anti-4-1BB mAb efficacy was investigated first, as it has been shown to directly promote T-cell development of CTL effector function and is also known to have agonistic effects on NK cells, making it an attractive agent to use in neuroblastoma, where MHC downregulation may make NK cell responses important in generating effective immunity (12, 27–30). Mice with small, palpable (1-2 mm diameter) Neuro2a tumors were treated with systemic anti-4-1BB mAb, resulting in complete tumor regression and long-term survival (>180 days) in approximately 60% of the mice treated (Fig. 1A). Interestingly, effective therapy was only achieved when mice received mAb therapy after tumors were well established, and not if therapy was given soon after tumor inoculation, before development of palpable tumors (Fig. 1B). Furthermore, mice cured of Neuro2a by anti-4-1BB mAb treatment acquire long-term immunity against rechallenge. The majority of mice (~80%) receiving 2 × 10^6 cells subcutaneously 180 days after initial inoculation showed increased survival compared with naive age-matched controls (P = 0.0004). Data represent examples of at least 2 experiments where n = 5 mice per group.

**Neuro2a tumor resolution after anti-4-1BB treatment requires CD8^+ T cells and NK cells, but not CD4^+ cells**

In vivo depletion of CD8^+ T cell and NK cell populations had minimal effect on the rate of Neuro2a tumor growth (data not shown), but completely abrogated the therapeutic effects of anti-4-1BB (Fig. 2A). In contrast, mice lacking CD4^+ T cells remained completely sensitive to anti-4-1BB therapy. To further explore the effector population mediating immunotherapy, treatment was delayed, thus avoiding complete resolution of tumor and allowing tumor excisions for immunohistochemical staining. A significant CD8^+ and NK lymphocyte infiltrate was observed in the majority of tumors taken from treated mice, as was scanty CD4^+ infiltration in treated but not untreated mice (Fig. 2B).

**mAb against CD40 and CTLA-4 are also effective in the Neuro2a model**

Antibodies targeting both CD40 and CTLA-4 were also shown to be therapeutic in the Neuro2a model (P < 0.0001 for both; Fig. 3A); with efficacy comparable with that observed with anti-4-1BB mAb. None of the neuroblastoma cell lines investigated showed significant expression of 4-1BB, CD40, or CTLA-4 (Supplementary Fig. S1), and there was no in vitro evidence of either direct cell death...
Immunomodulatory mAb alone are not effective against more aggressive neuroblastoma tumors

Next, the \textit{in vivo} activity of these 3 immunomodulatory mAb was investigated in the more aggressive, AgN2a and NXS2 neuroblastoma models. Here, single-agent immunomodulatory mAb therapy with anti-CD40, anti-CTLA4, or anti-4-1BB resulted in only marginal slowing of tumor growth (Fig. 3B and C). Although occasionally long-term survival was observed, there was no significant overall survival advantage in these models.

Combination of immunomodulatory mAb with peptide vaccination provides effective therapy against aggressive neuroblastoma tumors

It was postulated that provision of extra tumor antigen in the form of peptide vaccination may enhance the therapeutic effects of the immunomodulatory mAb. The survivin peptide GWEPDDNPI is a nonamer with predicted high-affinity binding to murine class I H2-Kk (31) and has been shown by others to be biologically active, where T-cell responses targeting this epitope have contributed to therapeutic immunity in mice treated with a cytokine-transfected Neuro2a cellular vaccine therapy (26). qPCR confirmed survivin expression in the 3 neuroblastoma cell lines with little expression observed in normal murine tissues other than the spleen and gut as previously reported (Fig. 4A).

Anti-CTLA-4 mAb given with IFA-emulsified GWEPDDNPI peptide (but not control peptide) resulted in effective therapy in both the AgN2a and NXS2 models (Fig. 4B). The combination therapy significantly prolonged survival compared with either GWEPDDNPI peptide (\(P = 0.013\) and \(P = 0.0353\) for AgN2a and NXS2, respectively) or anti-CTLA-4 mAb alone (\(P = 0.047\) and \(P = 0.0346\) for AgN2a and NXS2, respectively) by abrogating tumor growth (Supplementary Fig. S3 and data not shown). Again, therapy was dependent on the presence of CD8\(^{+}\) and NK cells (Fig. 4C), and long-term survivors from the anti-CTLA-4/GWEPDDNPI peptide treatment groups were protected against NXS2 tumor rechallenge (\(P < 0.0001\); Fig. 4D). Interestingly, despite similar efficacy to anti-CTLA-4 mAb in the Neuro2a model, neither anti-CD40 nor anti-4-1BB mAbs were found to provide effective therapy when combined with the GWEPDDNPI peptide in the more aggressive tumor models (Fig. 4B). These data suggest that the GWEPDDNPI peptide in combination with certain immunomodulatory mAb is able to generate effective antitumor immunity in aggressive neuroblastoma models.

Splenocytes from mice treated with anti-CTLA-4 and GWEPDDNPI show tumor-directed killing

Splenocytes from tumor-inoculated mice receiving anti-CTLA-4/GWEPDDNPI peptide treatment \textit{in vivo} showed effective lysis of tumor cells in CTL-killing assays \textit{ex vivo} (Fig. 5A). In contrast, splenocytes from naïve mice similarly stimulated \textit{in vitro} were inefficient at killing tumor cells (Fig. 5A). Lysis was observed against the cell line that the mice
had been inoculated with (NXS2) and additionally the AgN2a neuroblastoma cell line (Fig. 5A). This may be because both cell lines express survivin, or because immunity has been generated against other shared antigens. Lytic activity was not significantly increased when tumor cells or splenocytes were pulsed with the GWEPDDNPI peptide during the in vitro assay (Fig. 5B and Supplementary Fig. S4). Initially it was hypothesized that the relative high expression of survivin on these tumors cells may mean that survivin-specific lysis was already maximal. However, as splenocytes pulsed with GWEPPDNPI peptide also did not show enhanced lysis, it is more likely that epitope spreading to other target antigens is involved in the antitumour response observed.

**Combined therapy with anti-CTLA-4 mAb and GWEPPDNPI peptide generates antisurvivin immunity**

Having established that splenocytes from mice treated with anti-CTLA-4/GWEPPDNPI peptide had antitumor lytic activity ex vivo, we next wished to determine whether these mice had developed survivin-specific immune responses. Fourteen days after therapy with control peptide, GWEPPDNPI peptide, anti-CTLA-4 or anti-CTLA-4/GWEPPDNPI peptide, CFSE-labeled, GWEPPDNPI-pulsed target, and control peptide-pulsed nontarget splenocytes were adoptively transferred into treated mice. Specific depletion of target cells was observed 24 hours later only in mice treated with the combined anti-CTLA-4/GWEPPDNPI peptide therapy and not in mice that...
received the control or single-agent therapy (p = 0.05; Fig. 5C). This shows that anti-CTLA-4 combined with GWEPPDNPI peptide vaccine was able to generate anti-survivin immunity. Furthermore, following 6 day ex vivo stimulation with GWEPPDNPI-pulsed irradiated splenocytes, CD8+ T cells from mice treated with anti-CTLA-4 and GWEPPDNPI showed IFN-γ production after 5-hour incubation with NXS2 cells. This was in comparison with mice receiving control peptide, GWEPPDNPI or anti-CTLA-4 alone (P = 0.0138, 0.0017, and 0.0172, respectively; Fig. 5D).

**Effective anti-CTLA-4 and GWEPPDNPI therapy was not observed in the absence of tumor**

In the absence of tumor, splenocytes from mice that had received anti-CTLA-4/GWEPPDNPI therapy for 35 days in vivo were unable to generate antitumor cell lysis in CTL-killing assays against either NXS2 or AgN2a neuroblastoma cells (Fig. 6A). Alongside this, in vivo depletion of the target GWEPPDNPI-pulsed population was absent when labeled splenocytes were adoptively transferred into mice treated as previously described, but without prior tumor inoculation (Fig. 6B). Finally, prophylactic administration of anti-

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Figure 5. Anti-CTLA4 and GWEPPDNPI peptide immunotherapy generates tumor-directed cytotoxicity. Splenocytes from NXS2-inoculated mice receiving anti-CTLA-4/GWEPPDNPI peptide immunotherapy as previously described were harvested after 35 days and restimulated alongside splenocytes from naïve mice with GWEPPDNPI-pulsed, irradiated splenocytes for 6 days. Target cells labeled with 51Cr were incubated with splenocytes (effectors) at 50:1, 10:1, and 1:1 effector:target (E:T) ratio for 5 hours under tissue culture condition. A, splenocytes from mice treated with anti-CTLA-4/GWEPPDNPI peptide but not from naïve mice showed lytic activity against NXS2 and AgN2a tumors (P < 0.05). B, NXS2 cell lysis was not enhanced when the NXS2 cells were pulsed with a control or GWEPPDNPI peptide. C, fourteen days after therapy, CFSE-labeled peptide-pulsed splenocytes were adoptively transferred into NXS2-inoculated mice treated with control peptide, GWEPPDNPI, anti-CTLA-4 alone, or anti-CTLA-4/GWEPPDNPI peptide. After 24 hours peptide-specific killing was observed only with anti-CTLA-4/GWEPPDNPI treatment, as determined by the T:NT ratio. D, splenocytes from C restimulated as described above were incubated with unlabeled NXS2 target cells at a 50:1 E:T ratio for 5 hours in the presence of brefeldin A under tissue culture conditions. Using intracellular flow cytometry, IFN-γ production was detected in CD8+ T cells in splenocytes from anti-CTLA-4/GWEPPDNPI-treated mice only following in vitro restimulation with GWEPPDNPI. Data represent examples of at least 3 experiments where n = 3 mice per group. Error is expressed as SEM. *, P < 0.05; n.s., not significant.
CTLA-4/GWEPDDNPI peptide (before tumor inoculation) was not protective against tumor growth and did not achieve long-term survival (Fig. 6C). These data therefore suggest that the presence of tumor is required to generate an effective antitumor response in vivo.

Taken together, these data suggest that anti-CTLA-4 and survivin peptide vaccination can be used to generate potent antitumor immunity in these aggressive neuroblastoma models. Despite the fact that this therapy provides tumor antigen, generation of effective antitumor immunity seems dependent on the presence of tumor per se. Whether tumor is required to provide other tumor antigens, or to provide some form of "danger" signal is unclear, but is the subject of ongoing investigation.

Finally, it should be noted that in all of the above experiments, in the absence of tumor progression, treated mice remained well with no overt clinical or histologic signs of toxicity from the immunotherapy. In particular, there was no obvious weight loss, gut toxicity, or histologic evidence of colitis (data not shown).

Discussion

Successfully treating metastatic neuroblastoma remains a major challenge of pediatric oncology. Using the immune system to target tumor is an attractive treatment option for these children. Although many immunotherapeutic strategies have proved effective in preclinical neuroblastoma models, relatively few have entered clinical trials. To date, most of these have been "passive" immunotherapies, such as anti-GD2 mAb therapy (32). In 2010, the United Stated Children's oncology Group reported a large randomized control trial that showed a significant 2-year event-free survival benefit (66 vs. 46%) in children who had received anti-GD2–based immunotherapy in addition to standard high-risk neuroblastoma therapy (33). Although clearly encouraging, data from this study are still relatively immature and a large proportion of children still die from their disease.

The advantage of "active" immunotherapies is that they potentially achieve long-term immunity and tumor protection. Immunomodulatory mAb offers a potential mechanism of enhancing circulating tumor-specific T cells in patients to achieve protective antitumor immunity. For effective activation, T cells must not only engage their specific antigen but also a number of costimulatory molecules (34). Such molecules are usually absent on the surface of neuroblastoma cells themselves (35, 36) and although tumor antigens may be cross-presented by professional antigen-presenting cells, expression of costimulatory...
molecules by these cells is likely to be low in the relatively noninflammatory tumor environment (37). Furthermore, neuroblastoma cells themselves may abrogate expression of costimulatory molecules (e.g., CD40) on dendritic cells (38). Therefore, despite the presence of potentially immunogenic tumor antigens, antitumor T-cell responses may be suboptimal or even rendered tolerogenic (39). Transfection of costimulatory ligands (CD80, CD86, 4-1BB, CD40L) into murine neuroblastoma cell lines may overcome this defect, providing effective prophylactic tumor vaccination (40). Immunomodulatory mAb are a more practical and effective way of achieving this, either by acting agonistically, functioning as surrogate ligands, or by blocking immune-regulating molecules such as CTLA-4. Here, we show that a range of such mAb provides very effective therapy and may be successfully combined with other immunotherapies such as peptide vaccines. Unlike many other immunotherapies, such as cellular vaccines, these mAb do not need to be tailored to individual tumors or patient HLA types. This makes them a more attractive therapy to take forward to the clinic.

In the Neuro2a model, antibodies targeting CTLA-4, 4-1BB, or CD40, all provide effective therapy. The timing of mAb delivery is crucial and is only efficacious if given in a therapeutic rather than prophylactic setting. Although perhaps counterintuitive, this phenomenon has been noted previously when immunostimulatory mAb therapies are used in other tumor models, suggesting that the antitumor immune response generated is dependent on the presence of tumor antigen, and therapy is limited when mice are treated with a low tumor burden (13). In these models, rapid in vivo tumor growth kinetics result in there being a relatively narrow therapeutic window, between giving the mAb too early (when there may be insufficient tumor antigen) and too late (when there is insufficient time to mount an effective immune response before the animal succumbs to disease). In more aggressive neuroblastoma models (NXS2 and AgN2a), we were unable to achieve effective therapy with mAb alone. It was unclear whether this was simply due to the faster in vivo growth of these tumors, or because these tumors were less intrinsically immunogenic. We postulated that in either instance, therapy may be enhanced by combining the immunostimulatory mAb with a peptide vaccine targeting the tumor antigen survivin. Survivin is a member of the inhibitor of apoptosis family of proteins and is attractive as a target antigen for a number of reasons. Not only is it almost universally expressed in human high-risk neuroblastoma, it is also expressed in a number of other pediatric malignancies and many adult tumors, but with little expression in normal tissues (7). Expression of survivin seems to confer proangiogenic and antiapoptotic properties giving survival advantage to the tumor. These properties would be lost if the antigen were to be down-regulated as a means of immune escape (41). In addition, as previously mentioned, spontaneous T-cell responses to survivin have been recognized in children with neuroblastoma, suggesting the clinical relevance of the antigen (7). Therefore, there is already interest in developing clinical vaccines and immunotherapies targeting this antigen, with a number of adult phase I studies of survivin vaccine-based therapies having been reported (42, 43). In general, minimal epitope peptide vaccines given alone have not proved successful as cancer immunotherapeutics (44). In accordance with this, we did not observe any survival advantage in mice treated with survivin peptide alone. However, we found that combining survivin peptide with immunomodulatory mAb provided highly effective therapy. In addition, broader immune responses were potentially generated, targeting other antigens released when tumor cells were killed by antisurvivin CTLs, and boosted by circulating immunomodulatory mAb. Although initial peptide-specific responses were observed, later on T-cell responses did not seem to be predominantly directly against survivin, suggesting epitope spreading. The generation of a broader immune response, directed against nonvaccine antigens is potentially clinically advantageous, and is the focus of ongoing investigation within our laboratory.

There has been recent concern that survivin expression by activated CTLs may actually render them targets, and subject to fratricide, limiting the potential of any survivin-directed immunotherapy (45). The potent therapeutic effects, we observed, would argue against this being a major problem in vivo and the fact that mice successfully treated with mAb are left protected from rechallenge with the same tumor, suggests that sustained, functional, protective immunity is generated by this combination therapy.

In our experiments, the most promising mAb seemed to be anti-CTLA-4, and it is mAb targeting this molecule that have so far shown most promising in adult clinical trials (16). To date, the majority of clinical trials have been in patients with melanoma. Like melanoma, neuroblastoma is derived from neuroectodermal tissue, and immunologically there are many parallels and common tumor antigens. Although many of the melanoma studies have included peptide vaccination in addition to anti-CTLA-4, this did not confer additional benefit in the phase III study (16). This contrasts with our experience in murine models, and may be because there was preexisting tolerance to the epitopes of the chosen vaccine, or because in these patients, there was sufficient endogenous tumor antigen.

Despite the fact that our experiments showed superior efficacy in the context of increasing tumor burden, it is unlikely that immunotherapy alone will provide effective therapy in children with bulky disease. For most immunotherapies, there is increased likelihood of success in the context of low tumor burden, or minimal residual disease (MRD). Children that initially respond well to chemotherapy often harbor microscopic MRD resulting in eventual tumor relapse. Before relapse children are relatively well and can potentially receive a MRD-directed regime such as immunotherapy. In this context, there would be rationale for delivering the immunomodulatory mAb with tumor antigen in the form of peptide vaccine.

There is reservation about the usefulness of T-cell–mediated immunotherapies in children with neuroblastoma,
given that these tumors frequently exhibit low or absent levels of MHC class I (46). However, there is clinical evidence from other experimental T-cell–mediated therapies that this may not be a barrier for effective therapy. Russell and colleagues reported a phase I study of a vaccine consisting of an allogeneic human neuroblastoma transfected with IL-2 and lymphoatxin, a chemokine with T-cell attractant properties. Evidence of tumor-specific T-cell responses was reported. Of 28 children treated, there were 4 complete tumor responses to the vaccine (2 sustained for more than 4 years), 2 partial responses, and 5 children with stable disease (47). This suggests that, providing CTL are adequately primed, only very low levels of surface MHC/antigen are needed to render tumor cells targets for killing. In addition, surface expression of MHC class I on neuroblastoma is upregulated in the context of IFN-γ (48), which is potentially released by tumor-perforating lymphocytes as part of the antitumor immune response. This has been found to be the case with the B16-F10 metastatic melanoma model where expression of both MHC class I and II is increased on tumor cells following anti-4-1BB therapy (49). Although we saw no signs of obvious toxicity in mice treated with any of the mAb or peptide therapies, anti-CTLA-4 mAb have been associated with significant toxicity in patients, with severe (grade 3 or 4) immune-related adverse events (e.g., colitis, dermatitis, hypophysitis) in up to 10% to 15% of the patients (16). Remarkably, immune-related adverse events have tended to correlate with therapeutic benefit (50). The vast majority resolved with either withdrawal of mAb therapy or with corticosteroid treatment, the latter of which was shown not to abrogate therapeutic benefit. There is no reason why toxicity should be worse in children than adults, but clearly introduction of these agents into pediatric clinical trials should proceed cautiously, and should build on the adult experience in terms of prevention and management of this spectrum of toxicities, which would be unfamiliar to most pediatric oncologists. There would also be potential concern about targeting prevention and management of this spectrum of toxicities, and therefore also be explored. All of these risks and potential toxicities have to be balanced against the significant toxicity of current neuroblastoma therapies and the high mortality of the disease.

Finally, although we did not find any evidence of an expression of costimulatory molecules or direct effects of the immunostimulatory mAbs on these neuroblastoma cell lines, there is some published data suggesting that CD40 is expressed by human neuroblastoma (35). In other tumors in which CD40 is expressed by the tumor, there does seem to be a direct killing effect, in addition to indirect effects on immune system (13). It is therefore possible that additional therapeutic mechanisms may be seen in patients that we have not observed in our murine models.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E.L. Williams, P.W. Johnson, M.J. Glennie, J.C. Gray
Development of methodology: E.L. Williams, J.C. Gray
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