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Immunology and Immunotherapy of Neuroblastoma

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Abstract

Purpose—This review demonstrates the importance of immunobiology and immunotherapy research for understanding and treating neuroblastoma.

Principal results—The first suggestions of immune system-neuroblastoma interactions came from in vitro experiments showing that lymphocytes from patients were cytotoxic for their own tumor cells and from evaluations of tumors from patients that showed infiltrations of immune system cells. With the development of monoclonal antibody (mAb) technology, a number of mAbs were generated against neuroblastoma cells lines and were used to define tumor associated antigens. Disialoganglioside (GD2) is one such antigen that is highly expressed by virtually all neuroblastoma cells and so is a useful target for both identification and treatment of tumor cells with mAbs. Preclinical research using in vitro and transplantable tumor models of neuroblastoma has demonstrated that cytotoxic T lymphocytes (CTLs) can specifically recognize and kill tumor cells as a result of vaccination or of genetic engineering that endows them with chimeric antigen receptors. However, CTL based clinical trials have not progressed beyond pilot and phase I studies. In contrast, anti-GD2 mAbs have been extensively studied and modified in pre-clinical experiments and have progressed from phase I through phase III clinical trials. Thus, the one proven beneficial immunotherapy for patients with high-risk neuroblastoma uses a chimeric anti-GD2 mAb combined with IL-2 and GM-CSF to treat patients after they have received intensive cyto-reductive chemotherapy, irradiation, and surgery. Ongoing pre-clinical and clinical research emphasizes vaccine, adoptive cell therapy, and mAb strategies. Recently it was shown that the neuroblastoma microenvironment is immunosuppressive and tumor growth promoting, and strategies to overcome this are being developed to enhance anti-tumor immunotherapy.

Conclusions—Our understanding of the immunobiology of neuroblastoma has increased immensely over the past 40 years, and clinical translation has shown that mAb based immunotherapy can contribute to improving treatment for high-risk patients. Continued immunobiology and pre-clinical therapeutic research will be translated into even more effective immunotherapeutic strategies that will be integrated with new cytotoxic drug and irradiation therapies to improve survival and quality of life for patients with high-risk neuroblastoma.

Keywords

neuroblastoma; immunology; immunotherapy; microenvironment; anti-GD2

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Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood, and 45% of patients have high-risk tumors, nearly all of which are metastatic (stage 4) when diagnosed (1–4). Although outcome has steadily improved over the past 20 years, event-free survival (EFS) is still only 45% for patients with high-risk, metastatic disease (5–7). This review will focus on this group of patients since those with low- and intermediate-risk disease are currently effectively treated with surgery alone or surgery and modest-dose chemotherapy and so are not likely to receive immunotherapy in the future.

Initial evidence suggesting immune responses to neuroblastoma was provided in 1968 when blood leukocytes, which were 50–70% lymphocytes, from nine children with neuroblastoma were reported to inhibit colony formation by neuroblastoma cells that had been cultured for 10–30 days prior to testing (8). These lymphocytes inhibited colony formation by both autologous and allogeneic neuroblastoma cells but did not affect growth of fibroblasts from the same donors. Plasma from these patients also was reported to inhibit tumor cell colony formation in the presence of complement (8). In this same time, primary tumors were reported to contain leukocytes (9, 10), and some localized and metastatic neuroblastomas were reported to regress spontaneously (11–13). Together, these studies supported the hypothesis that the immune system could develop an anti-neuroblastoma response. However, the technology available at the time was not sufficient to define the cellular or molecular basis for the observations, and so definitive conclusions could not be reached.

Current “standard” therapy for high-risk patients, given in sequence, consists of 1) intensive induction chemotherapy and surgery; 2) myeloablative consolidation chemotherapy, autologous hematopoietic progenitor cell transplantation, and local irradiation; and 3) 13-cis-retinoic acid (cisRA) combined with IL-2, GM-CSF, and anti-disialoganglioside (GD2) mAb ch14.18, which targets tumor cells (6, 7). Although EFS was improved by adding ch14.18, IL-2 and GM-CSF immunotherapy to cisRA, approximately 40% of patients still relapse during or after this therapy (7). Development of new and more effective immunotherapy strategies for treating minimal residual disease and possibly for treating clinically measurable disease will be based upon improved understanding of interactions between tumor cells and the immune system and upon maximizing anti-tumor cell immune responses while minimizing or blocking pro-tumor and immunosuppressive immune responses.

This paper will review anti-tumor and pro-tumor (Yin and Yang) functions of the immune system in the context of neuroblastoma. The development and implementation of clinical immunotherapy with mAb ch14.18 has been successful as demonstrated in a phase III randomized study (7). However, anti-neuroblastoma T cell therapy trials, including those testing vaccines and adoptive cell therapy, are in early development and not yet proven to be beneficial. Pro-tumor functions (Yang) of the immune system in the tumor microenvironment and their negative impact upon immunotherapy of neuroblastoma are just beginning to be recognized, and further improvement of immunotherapy will need to address the tumor microenvironment.

Cytotoxic T Lymphocytes

The discovery that human cytotoxic T lymphocytes (CTLs) recognize a peptide presented by MHC class I molecules on autologous melanoma cells (14–16) opened the way for defining T cell targets on tumor cells. The peptide recognized in these experiments was derived from a protein encoded by the melanoma antigen-1 gene (MAGE-1, which was subsequently renamed MAGEA1). This gene, which is expressed by cancer cells and but not by normal cells with the exception of testis, was found to be a member of a large multi-gene family that

has been termed the cancer/testis antigen family (16–18). Neuroblastoma cell lines and tumors express MAGE-1/MAGEA1, MAGE-3/MAGEA3, MAGE-6/MAGEA6, and NY-ESO-1/CTAG1B (19) (20–22). Although these genes are expressed by human neuroblastoma cells, natural immune responses to their peptides have not been reported, and clinical vaccine trials have not been performed. There is one report that CTLs against peptides from MAGEA1, MAGEA3, and CTAG1B can be generated in vitro using T cells from normal donors and that these CTLs are cytotoxic against HLA class I compatible human neuroblastoma cells (23).

Clinical and pre-clinical studies have identified peptides from survivin, which is an inhibitor of apoptosis protein, as targets for CTLs. Survivin is expressed by many malignancies, including neuroblastoma (24, 25). In a study of nine patients with high-risk neuroblastoma, T cells specific for survivin were detected in blood at diagnosis by tetramer analysis, and circulating survivin-specific CTLs, after stimulation with survivin in vitro, were cytotoxic for autologous and HLA-compatible neuroblastoma cells. However, by immunohistochemistry, tumor-infiltrating T cells were few or absent in 26 of 26 tumors (25). Survivin specific CTLs from patients have been induced in vitro by monocyte-derived dendritic cells transfected with neuroblastoma cell line mRNA (26). Using a syngeneic mouse neuroblastoma model, protective tumor immunity that at least in part was due to CTLs that recognized survivin peptides was induced by immunization with Neuro-2A cells transfected with IL-21 (27). With the NXS2 murine neuroblastoma model, oral vaccination with a survivin DNA minigene was associated with increased target cell lysis, increased presence of CD8(+) T-cells at the primary tumor site, and enhanced production of pro-inflammatory cytokines. Therapeutic vaccination led to complete neuroblastoma eradication in over 50% of immunized mice and surviving mice showed an over 80% reduction in primary tumor growth upon re-challenge (28).

Pre-clinical studies have demonstrated that tyrosine hydroxylase and MYCN proteins, which are relatively specific for neuroblastoma cells compared to normal cells, include peptides that can be targets for CTL. Vaccination of mice with tyrosine hydroxylase DNA minigenes can induce CTLs, eradicate established primary NXS2 neuroblastoma tumors, and inhibit spontaneous metastases without induction of autoimmunity (29, 30). MYCN derived peptides have been reported to generate MHC class I restricted human CTL in vitro that lyse MYCN amplified human neuroblastoma cells (31).

Pre-clinical and clinical immunotherapy studies have used neuroblastoma cells that have been transduced with cytokine genes to provide multi-antigen vaccines without identifying the specific target antigens. Murine Neuro-2A neuroblastoma cells that were transfected with IL-2 were less tumorigenic than unmodified parent cells, induced protective immunity against parent cells, and prolonged survival of mice with established Neuro-2A tumors. These functions were dependent upon CD8+ T cells (32). The Neuro-2A model also was used to demonstrate that transfection of tumor cells with GM-CSF and IFN γ genes significantly improved their ability to induce protective immunity against liver tumors (33).

Early phase clinical trials have used cytokine transfected autologous or allogeneic neuroblastoma cells for immunization. IL-2 transfected autologous neuroblastoma cells induced antitumor immune responses in patients with neuroblastoma manifested by IgG antitumor antibody and increased CTL killing of autologous tumor cells. Clinically, five of ten patients had tumor responses (one complete and one partial response and three stable disease), and four of these five were shown to have coexisting antitumor cytotoxic activity (34). In a second study, 12 patients were immunized with an IL-2 transfected allogeneic neuroblastoma cell line, and although none showed any increase in direct cytotoxicity against the immunizing cell line, one had a partial response, 7 had stable disease, and 4 had

progressive disease (35). Another phase I study used the same allogeneic neuroblastoma cell line transfected to secrete both IL-2 and lymphotactin/XCL1, a chemokine for T cells. Among 21 patients with relapsed or refractory neuroblastoma, the vaccine increased CD4+ T cells, NK cells, eosinophils, and serum IL-5. Six patients had increased NK cytolytic activity, and 15 made IgG antibodies that bound to the immunizing cell line. Measurable tumor responses included complete remission in two patients and partial response in one patient (36). In a phase I study, seven patients received lymphotactin/XCL1 and IL-2-secreting autologous neuroblastoma cells, and this was associated with increased tumor recognition as measured by enzyme-linked immunosorbent spot (ELISPOT) assays (two patients had IFN γ and three had IL-5 responses). Clinical responses did not occur (37). In the most recent study, 13 patients who were presumed to have minimal residual disease (8 in first remission and 5 after treatment for recurrent NB) received an autologous neuroblastoma cell-IL-2 vaccine. ELISPOT assays for IFN γ and IL-5 demonstrated that vaccination produced a rise in circulating CD4 and CD8 T cells responsive to stimulation by autologous tumor cells. Median EFS was 22 months for patients in first remission, and three months for all others. Four patients treated in first remission remain alive and three are without disease recurrence (29+, 33+ and 41+ months). It is not possible to determine if the vaccine enhanced EFS from this small study (38).

Strategies aiming to generate CTLs must take into account mechanisms by which neuroblastoma cells may avoid immune elimination. These include decreased expression of peptide presenting HLA class I molecules by tumor cells, which can impair target peptide recognition by CTLs (25, 39–41). Also, neuroblastoma cells express low levels of antigen processing genes, including LMP-2, LMP-7, and TAP-1, which are necessary for preparation of peptides from proteins for presentation by HLA class I molecules to CTLs (42, 43). Neuroblastoma cells may suppress activation of CTLs as they have been reported to produce soluble MICA, which inhibits activation of NK cells and T cells via their NKG2D receptors (44) and 4Ig-B7-H3/CD276, which inhibits activation of NK cells (45) and T cells (46). Neuroblastoma cells also induce monocytes to release HLA-G, which suppresses both CTL and NK mediated cytotoxicity by interacting with inhibitory receptors or inducing apoptosis via CD8 ligation or the Fas-FasL pathway (47, 48). Finally, as discussed below, neuroblastoma cells recruit monocytes where they differentiate in the tumor microenvironment into M2 polarized macrophages to secrete potentially immunosuppressive cytokines including IL-6, IL-10, and TGF β 1 (49). Thus, effective CTL anti-tumor responses require that these escape mechanisms be evaluated and, if present, be overcome.

NKT cells

V α 24-invariant (type I) natural killer T cells (NKTs) react to self- and microbial-derived glycolipids presented by the monomorphic HLA class-I-like molecule CD1d. They express an invariant TCR α -chain, V α 24-J α 18 which is preferentially paired with V β 11 (50). NKT cells are cytotoxic for tumor cells only if the latter expresses CD1d, and neuroblastoma cells do not express this surface molecule. However, NKT cells, if stimulated, secrete IL-2, which activates NK cells, and so indirectly NKT cells can mediate cytotoxicity against neuroblastoma cells (51). NKT cells were found to infiltrate primary neuroblastomas from patients with metastatic disease, which was due to tumor cell secretion of the chemokine MCP-1/CCL2. CCL2 expression by tumors was found to be inversely correlated with MYCN proto-oncogene amplification and expression, and MYCN-high/CCL2-low expression was associated with the absence of NKT cells in primary tumors (52). Further studies showed that MYCN down-regulates expression of CCL2 and so impacts localization of NKT cells into neuroblastoma tumors. This was the first demonstration that an oncogene can regulate infiltration of immune system cells into the tumor microenvironment (53). A

possible role for NKT cells in mediating anti-tumor responses is suggested by the discovery that NKT cells can kill monocytes pulsed with tumor lysates. Cotransfer of human monocytes and NKT cells to neuroblastoma-bearing NOD/SCID mice decreased monocyte number at the tumor site and suppressed tumor growth compared with mice transferred with monocytes alone (54). Clinical therapy trials attempting to enhance NKT antitumor functions have not yet been performed in patients with neuroblastoma.

NK cells

A number of studies support the importance of NK cells in the immune response to cancer. Intra-tumoral NK cells have been demonstrated in colorectal carcinoma, gastric carcinoma, and squamous cell lung cancer, and, in general, patients whose tumors have the highest levels have the best survival (55–58). NK cell immunoglobulin receptor Fc γ RIIIa functional polymorphism correlates with response to the IgG1 anti-CD20 mAb rituximab in patients with non-Hodgkin's lymphoma and Waldenstrom's macroglobulinemia indicating the importance of NK mediated ADCC (59–61). Neuroblastoma cells often do not express surface HLA class I and II molecules and so may be much better targets for NK cells than for CTLs (40, 41, 62–64). Two recent studies suggest an anti-tumor role for NK cells in high-risk neuroblastoma. In one, killer immunoglobulin-like receptor (KIR) and HLA gene polymorphisms which interact to govern NK cell function correlated with disease progression and survival in patients with high-risk disease treated with autologous hematopoietic stem cell transplant (AHST). Those with a “missing ligand” KIR-HLA compound genotype had a 46% lower risk of death at three years after AHST compared to patients who possessed all ligands for inhibitory KIR (65). In another, patients with relapsed or refractory neuroblastoma had a significantly better response to the anti-GD2 immunocytokine hu14.18/IL-2 if they had a KIR-HLA ligand mismatch (66). To date, studies of the relationship of Fc γ RIIIa functional polymorphism to outcome in patients with neuroblastoma who were treated with the ch14.18 mAb have not been performed.

NK cells are activated to be cytotoxic and secrete IFN γ by IL-2. NK cytotoxicity is perforin-dependent or -independent. The perforin-dependent pathway includes perforin, granzymes, and granulysin, whereas the perforin-independent includes FASL and TRAIL. Perforin-dependent cytotoxicity may be triggered by natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46, the C-type lectin heterodimeric receptor CD94/NKG2C, 2B4 (CD244), NKG2D, and DNAM1 (67–69). NCR and DNAM1 have been demonstrated to be involved in NK cell cytotoxicity against neuroblastoma cells (70, 71).

IL-2 alone has been tested in phase I and II trials for patients with neuroblastoma, and, although immune effects were documented, no objective tumor responses were observed (72–74). Lenalidomide is an immune modulating drug that activates T cells to secrete IL-2, which in turn activates NK cell cytotoxicity and ADCC (75, 76). Clinical trials in children and adults demonstrated increased numbers of NK cells and cytotoxicity, decreased T regulatory cells, and increased secretion of IL-2, IL-15, and GM-CSF after 21 days of lenalidomide treatment (77, 78). In the phase I study of lenalidomide in children and adolescents with relapsed or refractory solid tumors no objective responses occurred, but the drug was well tolerated without reaching a maximum tolerated dose (77). Thus, lenalidomide may be useful for activating NK cells to enhance mAb immunotherapy of neuroblastoma.

Anti-Tumor Cell Monoclonal Antibodies

The development of monoclonal antibody (mAb) technology (79) ultimately resulted in creation of therapeutically effective anti-tumor cell mAbs including rituximab (anti-CD20), cetuximab (anti-EGFR), trastuzumab (anti-HER2), and ch14.18 (anti-GD2) (7, 80–83). Soon

after the technology became available, multiple different mAbs were produced that recognize neuroblastoma cell associated antigens (84–95). Some were used to develop immunocytology, which improved detection and quantification of neuroblastoma cells in bone marrow and blood compared to morphologic evaluation (96, 97). Immunocytology evaluation of bone marrow and blood during induction chemotherapy provided prognostic information (98). It also demonstrated that 20% of autologous marrows that were harvested for AHSCT had detectible tumor cells (98). To minimize the possibility of infusing tumorigenic cells with the autologous transplant (99), mAbs were used for ex vivo immunomagnetic removal (“purging”) of tumor cells from autologous bone marrow (100). Purged autologous bone marrow with no immunocytology detectible tumor cells was used for AHSCT in the phase III randomized study that established that consolidation with myeloablative chemoradiotherapy was more effective than with nonmyeloablative chemotherapy (5, 6). Immunocytology showed that only 1% of peripheral blood stem cell collections had detectible tumor cells, and a randomized phase III study demonstrated equivalent event-free survival when purged vs. non-purged PBSC were used for hematopoietic reconstitution (neither had detectible tumor cells by immunocytology) (Kreissman, et al., in preparation, 2011). Thus, both immunocytology and immunomagnetic ex vivo purging contributed to improving treatment for patients with high-risk disease.

The most significant contribution of mAbs to treatment of high-risk patients came from the discovery that neuroblastoma cells express a high level of disialoganglioside (GD2) and from the generation of mAbs to this surface molecule (88, 91, 93, 94, 101, 102). Initial clinical studies demonstrated that anti-GD2 mAbs could induce responses in patients with neuroblastoma (95, 103–105), probably due to complement dependent cytotoxicity and antibody dependent cellular cytotoxicity (ADCC) (106–108). IL-2 and GM-CSF were shown to enhance ADCC in vitro by activating cytotoxic natural killer cells and neutrophils (106, 109–112). A phase I/IB study tested the combination of IL-2 and murine anti-GD2 antibody 14G2A in patients with recurrent neuroblastoma (113, 114). Phase I studies also tested the chimeric anti-GD2 mAb ch14.18 in refractory or relapsed patients (115) and in patients in first response following myeloablative therapy and AHSCT (116). A subsequent phase I study evaluated the combination of GM-CSF, IL-2 and ch14.18 therapy combined with cisRA following myeloablative therapy and AHSCT and found the regimen to be tolerable (117). This progression of clinical trials culminated in the recently completed phase III randomized study of cisRA together with ch14.18, IL-2, and GMCSF vs. cisRA only for children with high-risk neuroblastoma who had a clinical response to induction therapy and myeloablative consolidation therapy/AHSCT. Immunotherapy after consolidation significantly improved EFS (66+/-5% vs. 46+/-5% at 2 years, P=0.01) and overall survival (86+/-4% vs. 75+/-5% at 2 years, P=0.02 (7). This was the first demonstration that antibody based therapy improves EFS and overall survival. Although EFS was improved by adding immunotherapy to cisRA, approximately 40% of patients still relapsed during or after this therapy (7). Additionally, the combination of ch14.18 with IL-2 and GM-CSF has significant toxicities, including neuropathic pain, fever without neutropenia, infection, hypokalemia, hypotension, and capillary leak syndrome. Thus, a search for new agents to combine with ch14.18 to improve efficacy and decrease toxicity is justified.

Antibody-cytokine fusion proteins (immunocytokines) combine the targeting ability of antibodies with the functional activity of cytokines, and such molecules may improve antibody-based therapy by delivering cytokines to the microenvironment to both activate effector cells and modulate the microenvironment. Immunocytokines, depending upon their molecular composition, could create a microenvironment that results in 1) complement dependent cytotoxicity against tumor cells; 2) ADCC against tumor cells mediated by activated neutrophils, natural killer cells, and/or monocytes/macrophages; and 3) attraction

of dendritic cells that process tumor associated antigens and then induce antitumor immune responses by T and/or B cells. To date, immunocytokine research has focused on ADCC mediated by NK cells and on induction of CTL. An anti-GD2/IL-2 immunocytokine eradicated hepatic metastases of neuroblastomas in SCID mice that had been reconstituted with human lymphokine (IL-2) activated killer cells (118–120). In contrast, the combination of monoclonal anti-GD2 antibody and IL-2 at doses equivalent to the immunocytokine only reduced tumor load. In a syngeneic murine model of GD2 expressing melanoma, targeting with an anti-GD2 antibody/IL-2 immunocytokine resulted in generation of CD8+ T lymphocytes that could eradicate tumor as well as prevent tumor growth (120). Based upon these data, phase I and II studies have tested a humanized anti-GD2/IL-2 immunocytokine (hu14.18/IL-2) in patients with refractory or relapsed neuroblastoma. In the phase I study of 27 patients, treatment with hu14.18/IL2 caused elevated serum levels of soluble IL-2 receptor alpha (sIL2R α) and lymphocytosis. There were no measurable complete or partial responses to hu14.18/IL2; however, three patients showed evidence of antitumor activity (121). In the phase II study, 39 patients with recurrent or refractory neuroblastoma were enrolled (36 evaluable). No responses were seen for patients with disease measurable by standard radiographic criteria (stratum 1) (n = 13). Of 23 patients with disease evaluable only by ¹²³I-metaiodobenzylguanidine (MIBG) scintigraphy and/or bone marrow histology (stratum 2), five patients (21.7%) responded; all had a complete response of 9, 13, 20, 30, and 35+ months duration. Grade 3 and 4 non-hematologic toxicities included capillary leak, hypoxia, pain, rash, allergic reaction, elevated transaminases, and hyperbilirubinemia, which were reversible within a few days of completing a treatment course. These results support further testing of hu14.18/IL2 in children with non-bulky high-risk neuroblastoma (122).

Another strategy for improving antibody-based immunotherapy is to identify drugs that both activate NK cells for ADCC and prevent immunosuppression by the tumor microenvironment. Lenalidomide is an immune modulating drug that activates T cells to secrete IL-2, which in turn activates NK cell cytotoxicity and ADCC (75, 76). Preclinical *in vitro* data and mouse xenograft studies have shown that lenalidomide can enhance ADCC mediated by NK cells with rituximab (anti-CD20) against lymphoma and chronic lymphocytic leukemia cells (123–125), with SGN-40 (anti-CD40) against multiple myeloma and chronic lymphocytic leukemia cells (126, 127), and with trastuzumab (anti-HER2/neu) and cetuximab (anti-EGFR) against solid tumor cell lines (128). A phase I trial in children and adolescents with refractory solid tumors demonstrated increased NK cell numbers and cytotoxicity, decreased T regulatory cells, and increased serum IL-2, IL-15, and GM-CSF after 21 days of lenalidomide treatment (77). Our pre-clinical research demonstrated that lenalidomide enhances IL-2-mediated activation of NK cells, prevents their suppression by IL-6 and TGF β 1, which are in the neuroblastoma microenvironment, and increases ADCC *in vitro* and in NOD/SCID mice with mAb ch14.18 (Xu, et al., submitted for publication). Based upon these clinical and pre-clinical data, a phase I trial to test lenalidomide in combination with ch14.18 in patients with refractory or relapsed neuroblastoma is being developed by the New Approaches to Neuroblastoma Therapy (NANT) consortium.

Adoptive Cell Therapy with T cells and NK cells

Adoptive cell therapy (ACT) for high-risk neuroblastoma has focused upon growing autologous T cells *ex vivo* and transfecting them with cDNA encoding chimeric antigen receptors (CARs) that ligate tumor cell surface antigens and also provide activating signals to the T cells (129). CARs usually use a single chain fraction variable (scFv) antibody-derived motif for recognizing a cell surface antigen; importantly, such recognition is independent of antigen processing or MHC-restricted presentation. Primary human T cells with a CD28-like CAR specific for GD2 had enhanced survival and proliferation upon receptor stimulation (130). T cells engineered to express an anti-GD2 CAR (scFv from mAb

14.G2a linked to TCR ζ) recognized and lysed GD2-expressing neuroblastoma cells and secreted IFN γ in an antigen-specific manner. However, functionality declined over time in vitro, and antigenic stimulation did not induce proliferation (131). It was then shown that EBV-specific T cells, which were transduced with the anti-GD2 CAR gene, could be expanded and maintained long-term in the presence of EBV-infected B cells. These T cells efficiently lysed both EBV infected cells and GD2 expressing cells (132). Next, it was shown in a clinical trial that enrolled 11 patients that infusion of these genetically modified cells was safe and was associated with tumor regression or necrosis in four of the eight evaluable patients (two responses, two stable disease) (133). T cells expressing a CAR that targets the L1-CAM molecule on neuroblastoma cells were engineered by fusing the scFv of mAb CE7 to human IgG1 hinge-Fc, the transmembrane portion of human CD4, and the cytoplasmic tail of the human CD3-zeta chain. Primary human CD8⁺ CTL clones expressing this CAR specifically lysed human neuroblastoma cells and secreted GM-CSF, TNF α , and IFN γ (134). A clinical study of six patients in which autologous CD8⁺ T cells with anti-L1-CAM CAR were tested showed no overt toxicities to tissues known to express L1-CAM. Persistence of the CTL clones in the circulation was short (1–7 days) in patients with bulky disease but longer (42 days) in a patient with limited disease whose tumor partially responded (135). Migration to sites of disease is essential, and since MYCN non-amplified neuroblastomas express the chemokine CCL2 (52), it was determined if engineering T cells to express an anti-GD2 CAR and CCR2b, a receptor for CCL2, enhanced their migration. Injection of such T cells intravenously into SCID mice with subcutaneous neuroblastoma demonstrated specific migration, intra-tumor T cell expansion, and enhanced anti-tumor effect compared to anti-GD2 CAR T cells without CCR2b (136). Finally, expression of constitutively active Akt in anti-GD2 CAR T cells increased their proliferation and cytokine production, provided resistance to apoptosis, rendered them relatively resistant to suppression by and conversion into regulatory T cells, and increased cytotoxicity for GD2 expressing neuroblastoma cells in vitro (137).

Adoptive cell therapy with NK cells alone and especially combined with mAbs has therapeutic potential for a wide variety of human malignancies, including neuroblastoma. Recently, the ability to harvest large numbers of peripheral blood lymphocytes by leukapheresis, deplete alloreactive T cells, and activate the remaining NK cells with IL-2 has enabled haploidentical NK cell adoptive immunotherapy for AML in adults where remission in 5 of 19 poor-prognosis patients was associated with KIR mismatch (138). In a similar study, KIR-mismatched NK cell infusion was reported to be safe as post-remission consolidation therapy for children with AML with no relapses reported in the 10 patients treated (139). As noted above, patients with stage 4 neuroblastoma undergoing AHST who lacked a HLA ligand had a 46% lower risk of death and a 34% lower risk of progression at 3 years compared to those who possessed all ligands for their inhibitory KIRs. Among all KIR-HLA combinations, 16 patients lacking the HLA-C1 ligand for KIR2DL2/KIR2DL3 had the highest 3-year survival rate of 81%. In this study, survival was more strongly associated with "missing ligand" than with tumor MYCN gene amplification (65).

The inability to obtain large numbers of pure NK cells has limited testing the potential efficacy of NK adoptive cell therapy. A novel ex vivo expansion method for NK cells that uses an artificial antigen-presenting cell (aAPC) genetically engineered to express membrane bound IL-21, which signals primarily through STAT3, resolves this problem (140). The IL-21-expressing aAPCs promote sustained proliferation of mature NK cells without senescence by increasing the telomere length in the expanded cells. The IL-21 aAPC enable large-scale expansion of NK cells from a small volume of peripheral blood, sufficient to deliver multiple infusions of NK cells at high cell doses. These expanded NK cells display a highly-activated phenotype and participate in ADCC with mAb ch14.18 to kill neuroblastoma cells in vitro and in models of neuroblastoma in NOD/SCID mice. This

pre-clinical data, as well as pre-clinical and clinical data for lenalidomide, provide the basis for developing a phase I clinical trial by MD Anderson Cancer Center and the NANT consortium that will test autologous, ex vivo expanded and activated NK cells combined with lenalidomide and mAb ch14.18 in patients with refractory or relapsed neuroblastoma.

Microenvironment Promotion of Tumor Growth and Immunosuppression

The tumor microenvironment, whether primary or metastatic, is a complex milieu created by tumor cells interacting with normal host cells. Like the yin and yang concept, immune system cells can eliminate tumor cells, promote their growth and dissemination, and suppress antitumor immune responses (141, 142). The importance of the microenvironment in which neuroblastoma cells grow has not been widely appreciated for developing new therapies. The first suggestion that immune system cells promote neuroblastoma progression came from gene expression analysis of high-risk, metastatic neuroblastomas without MYCN gene amplification using Affymetrix® microarrays and TaqMan® low-density arrays (143). This research revealed that high expression of genes related to B lymphocytes, macrophages, and inflammation, including IL-6, IL-6R, IL-10, and TGFβ1, was associated with poor 5-year EFS (54, 143). Furthermore, high-risk primary neuroblastomas had CD68+ tumor-associated monocytes/macrophages (TAMs) expressing IL-6, and bone marrows with neuroblastoma metastases had CD33+CD14+ myelomonocytic cells, also expressing IL-6 (54). Experimentally, neuroblastoma cells stimulate blood monocytes to secrete IL-6, and TAMs stimulate the growth of neuroblastoma cells in NOD/SCID mice, at least partially via IL-6 secretion (54). TGFβ1 activates the SMAD2/3 pathway in human NK cells, which results in suppression of ADCC and IFNγ, TNFα, and GM-CSF secretion in vitro (144, 145). The possible role of B lymphocytes in neuroblastoma progression is not yet clear. However, in murine cancer models, B cells have been shown to secrete IL-10 as well as antibodies that form immune complexes in the microenvironment, and both contribute to M2 polarization of TAMs. (141, 146, 147) (142)

T regulatory cells (Tregs), which have a CD4+ CD25+ Foxp3+ phenotype, have not been evaluated in human neuroblastoma primary tumors or metastatic sites. These cells secrete TGFβ1 and IL-10, which are present in primary tumors from patients with stage 4 disease and which are known to suppress immune responses. Tregs have been studied using murine neuroblastoma models. Inhibition of CD25+ Tregs by treating mice with an anti-CD25 mAb partially depleted CD4+ Foxp3+ T cells and enhanced vaccine-induced immunity to challenge with Neuro-2A neuroblastoma cells (148). In another study, treatment of mice with an anti-CD4 mAb depleted 90% of Tregs and potentiated a Neuro-2A/IL-21 cell vaccine. 80% of mice did not develop disseminated disease after intravenous injection of tumor cells, and this was dependent upon CD8+ T cells (149). In a third study, mice bearing subcutaneous Neuro-2A (AGN2a subclone) neuroblastoma tumors were treated with total body irradiation followed by transplantation with syngeneic bone marrow combined with T cells. Mice receiving CD25+ depleted T cells had increased post-transplantation vaccine-induced immunity and survival (150).

Future Directions

New immunotherapeutic strategies for improving survival of patients with high-risk neuroblastoma will require continued improvement in understanding tumor and host cell biology. Biologic and immunobiologic knowledge will drive developmental therapeutics research with in vitro and in vivo models of neuroblastoma. Pre-clinical strategies that are promising and preferably supported by clinical data from studies of adults with cancer will be tested in early phase clinical trials. Some strategies may show convincing benefit in well-designed early phase studies, but most are likely to require randomized phase III trials to

definitively evaluate their efficacy. There are several important considerations in this discovery to development to delivery, bench to bedside to bench paradigm.

In discovery research, interactions of neuroblastoma and immune system cells that eliminate tumor cells (yin) and that promote immune suppression and tumor growth (yang) in primary and metastatic sites will be defined. Understanding how tumor cells create microenvironments that enhance their survival and dissemination while suppressing anti-tumor immune responses is particularly important. Well-characterized human neuroblastoma cell lines, which represent high-risk disease, provide models for in vitro and in vivo studies using human cells. Primary, short-term cultures of neuroblastoma cells, such as bone marrow metastases, allow studies of autologous interactions. Murine transgenic neuroblastomas that are MYCN amplified or non-amplified provide models of human neuroblastoma in immune competent mice, and these mice can be genetically manipulated to test hypotheses about immune system cells, pathways, and effector molecules. All models should be correlated with data from human primary tumors and bone marrow metastases to obtain clinical validation.

In the development phase, pre-clinical therapeutic research, which is based upon discovered immunobiology, tests new treatments against neuroblastoma cells in primary and metastatic sites. A key goal must be to successfully target the tumor microenvironment to tip the balance in favor of anti-tumor immunotherapy. This research uses the same models used in discovery to integrate immunobiology with immunotherapy. It is advisable to prioritize immunotherapeutic strategies that are most applicable for clinical delivery to patients and so, for example, to focus upon drugs and mAbs that already are approved or are likely to be approved by the FDA rather than agents that have not yet entered the clinical testing pipeline. Such prioritization will maximize the likelihood that agents shown to be effective in pre-clinical studies will be available for clinical testing. In this context, vaccine and adoptive cell therapy strategies, to be successful, will need to focus upon both efficacy in pre-clinical models and upon methods for efficient broad clinical application. An important aspect of this phase of immunotherapy research is the parallel development of assays for pharmacokinetics, pharmacodynamics, and tumor response, the latter seeking to provide an early surrogate of efficacy.

In the delivery phase, phase I clinical trials test the new immunotherapeutic strategy to determine appropriate dose and schedule, pharmacology and pharmacodynamics, and, within the context of such trials, anti-tumor activity. These trials generally require a consortium of several institutions with expertise in early phase trials for efficient implementation of protocols and accrual of patients with refractory or recurrent disease. Initial information about biomarker strategies should be obtained in early phase clinical studies. Definitive testing of the treatment and of the biomarkers is obtained from phase II and III clinical trials.

In developing new immunotherapies, it is important to consider their role in the overall treatment of high-risk neuroblastoma. Current dose intensive chemotherapy is likely to be at the limit of both anti-tumor efficacy and patient tolerance, and post consolidation therapy, even though including antibody immunotherapy, does not eradicate minimal residual disease (MRD) in many patients. Appropriate immunotherapy strategies will be developed that will contribute to improving induction, consolidation, and post-consolidation treatments. These new strategies will come from learning how to harness the power of the immune system to eliminate tumor cells and from learning how to integrate immunotherapy with non-specific and targeted drug therapies.

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Abbreviations

mAb	monoclonal antibody
CTL	cytotoxic T lymphocyte
GD2	disialoganglioside
EFS	event-free survival
NKT	V α 24-invariant (type I) natural killer T cell
NK cell	natural killer cell
ADCC	antibody dependent cellular cytotoxicity
AHSCT	autologous hematopoietic stem cell transplantation
TAM	tumor associated macrophage
Tregs	T regulatory cells

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