Neuroblastoma Arginase Activity Creates an Immunosuppressive Microenvironment That Impairs Autologous and Engineered Immunity

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Abstract

Neuroblastoma is the most common extracranial solid tumor of childhood, and survival remains poor for patients with advanced disease. Novel immune therapies are currently in development, but clinical outcomes have not matched preclinical results. Here, we describe key mechanisms in which neuroblastoma inhibits the immune response. We show that murine and human neuroblastoma tumor cells suppress T-cell proliferation through increased arginase activity. Arginase II is the predominant isoform expressed and creates an arginine-deplete local and systemic microenvironment. Neuroblastoma arginase activity results in inhibition of myeloid cell activation and suppression of bone marrow CD34+ progenitor proliferation. Finally, we demonstrate that the arginase activity of neuroblastoma impairs NY-ESO-1–specific T-cell receptor and GD2-specific chimeric antigen receptor–engineered T-cell proliferation and cytotoxicity. High arginase II expression correlates with poor survival for patients with neuroblastoma. The results support the hypothesis that neuroblastoma creates an arginase-dependent immunosuppressive microenvironment in both the tumor and blood that leads to impaired immunosurveillance and suboptimal efficacy of immunotherapeutic approaches.

Introduction

Neuroblastoma is the most common extracranial malignancy of childhood. Although the prognosis for low-risk neuroblastoma has improved, patients with high-risk disease have an extremely poor survival despite intensive multimodal treatment, including immunotherapy (1). Neuroblastoma is associated with an unique interaction with the immune system, clinically evidenced by patients who develop paraneoplastic opsoclonus–myoclonus syndrome and patients whose tumors spontaneously regress (2, 3). Over the last 10 years as the benefit of conventional therapies has been maximized, the focus has moved to enhancing an antineuroblastoma immune response. T cells are a major effector arm of the immune system and play a key role in the recognition and targeting of cancer cells. Subsequently engineered chimeric antigen receptor (CAR) T cells against the predominant neuroblastoma surface antigen GD2 demonstrated antineuroblastoma cytotoxicity in vitro and in murine models (4, 5). However, although preclinical studies demonstrate that T cells have the potential for antineuroblastoma activity, the clinical efficacy of immunotherapies has been controversial (6, 7). Immunotherapeutic approaches are reliant on an active immune system, therefore one likely hypothesis for their failure is that neuroblastoma creates an immunosuppressive microenvironment that inhibits autologous or adoptive immunity (8, 9).

The mechanisms underlying the immunosuppressive microenvironment in neuroblastoma are poorly understood. In this study, we identify the key role of neuroblastoma arginase activity in inhibiting both autologous and engineered antineuroblastoma immune responses.

Materials and Methods

Neuroblastoma patient samples

Blood and tumor samples were obtained from 26 patients with neuroblastoma treated at the Birmingham Children’s Hospital, Children’s Hospital Oxford, and Great Ormond Street Hospital (Supplementary Table S1). The samples were taken from patients with newly diagnosed neuroblastoma, at the time of diagnostic biopsy, before the start of treatment.

GD2+ tumor cell isolation

For isolation of GD2+ tumor cells from human and murine tumors, tumors were digested using Type II collagenase, labeled with anti-GD2-PE antibody, and bound to anti-PE–coated magnetic
beads (Miltenyi). Cells were purified according to manufacturer’s instructions (Miltenyi Biotec). Purity of GD2+ cells was >98% as confirmed by flow cytometry.

Neuroblastoma murine model immune characterization

After weaning, TH-MYCN mice were palpated for intra-abdominal tumors twice weekly. Mice with palpable tumors ranging in size between 5 and 20 mm in diameter were then humanly sacrificed. At sacrifice, unharmonized and heparinized whole blood, as well as tumor tissue and spleen were obtained for further ex vivo analyses. Tumor tissue was processed as above. Spleens were mechanically digested and heparinized whole blood was lysed with red blood cells lysis buffer (Qiagen). Tumor tissue, spleen, and blood cell suspensions were stained with anti-mouse Ly6C, Ly6G, F480, CD3, and GD2 antibody (Biolegend) on ice for 30 minutes. The expression of these markers was assessed by flow cytometry.

Arginase enzyme activity

The activity of arginase II present within neuroblastoma cell lines, sorted patient or murine cells, culture supernatants or plasma was determined by measuring the conversion of arginine into urea, as previously described (10).

Monocyte polarization assay

Peripheral blood was collected from healthy donors and monocytes were separated using a Lymphoprep gradient and enriched by negative selection using a Monocyte Isolation Kit II (Miltenyi). Monocytes were cultured in the presence or absence of neuroblastoma, neuroblastoma culture supernatants (50% of final volume), or patient plasma (50% of final volume) overnight, in low-adherent 24 plates (CoStar), and then treated with lipopolysaccharide (LPS; 10 ng/mL, Sigma) for 24 hours. The culture supernatants were harvested and analyzed for cytokine release. Cells were harvested and stained with anti-CD86 (Biolegend). Propidium iodide added to allow viable cells to be gated. The expression of CD86 on monocytes was assessed by flow cytometry.

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Murine myeloid cells were purified from spleens of healthy and tumor-bearing mice and from tumor tissue of neuroblastoma mice using flow cytometric sorting. The cells were cultured in R10% or arginine-free medium and treated with LPS for 24 hours. The culture supernatants were harvested and analyzed for cytokine release as before.

Statistical analysis

A Wilcoxon rank-sum test was used to determine the statistical significance of the differences in unpaired observations between two groups. All P values are 2-tailed and P < 0.05 was considered to represent statistically significant events.

Study approval

In accordance with the Declaration of Helsinki, patient samples were obtained after written, informed consent prior to inclusion in the study. Regional Ethics Committee (REC Number 10/H0501/39) and local hospital trust research approval for the study was granted for United Kingdom hospitals. The Institute of Cancer Research Ethics Committee approved all animal protocols in this study. Procedures were carried out in accordance with UK Home Office Guidelines.

Results

Neuroblastoma suppresses T-cell proliferation via arginase II expression and activity

T cells are the major effector arm of the endogenous anticaner immune response. However, neuroblastoma is able to develop and metastasize in patients, despite the immune system suggesting impaired immunosurveillance. We investigated the effect of neuroblastoma on T-cell immunity, using human cell lines as an established model of malignant neuroblastoma. Neuroblastoma cell lines were cultured with T cells from healthy donors. T-cell proliferation was significantly suppressed by the presence of neuroblastoma (Fig. 1A), even at low neuroblastoma: T-cell ratios. Using a Transwell assay, we identified that the T-cell suppression was non-contact-dependent (Fig. 1B). Cell line supernatant was examined for immunosuppressive molecules, and no detectable levels of IL10, IL4, and TGFβ were identified (data not shown), consistent with previous reports of an unknown alternative mechanism of immunosuppression mediated by neuroblastoma cells (9).

We have recently identified that another pediatric malignancy, acute myeloid leukemia (AML), creates an immunosuppressive microenvironment through altered arginase activity (10). Although AML is a hematologic malignancy and neuroblastoma a solid tumor, both cancers share a common association with pancytopenia, immune alteration, and bone marrow infiltration at diagnosis. Therefore, we hypothesized that neuroblastoma tumor cells similarly harness arginine depletion to suppress the immune response. Arginine is a semiessential amino acid, that is metabolized in mammalian cells, principally by the enzymes arginase I and II, and by inducible nitric oxide synthase (iNOS; ref. 11). As arginase I and II and iNOS may be responsible for inhibition of T-cell proliferation, we tested the functional relevance of each enzyme (12). By measuring the conversion of arginine into urea, we demonstrated that neuroblastoma cells have a measurable arginase activity, albeit lower than AML cell lines (24 μM/106 cells NB vs. 67.5 μM/106 cells AML; Fig. 1C). No increased production of reactive nitric oxide species was demonstrated (data not shown). Using the small molecules NOHA and L-NMMA to inhibit arginase and iNOS, respectively, we demonstrated that the immunosuppressive microenvironment is created by arginase activity alone (Fig. 1D). NOHA alone was able to rescue T-cell proliferation, with no effect from L-NMMA. Moreover, analysis of neuroblastoma culture supernatants showed that the concentration of arginine decreases significantly in the local microenvironment (Fig. 1E) and leads to downregulation of T-cell CD3ζ chain (Supplementary Fig. S1A). Addition of exogenous arginine led to moderate rescue of T-cell proliferation (Supplementary Fig. S1B). Both RT-PCR and Western blotting identified that neuroblastoma cell lines express arginase II as the main isoform (Supplementary Fig. S1C). With no measurable arginase I or iNOS protein expression (Supplementary Fig. S1E and S1F). The arginase activity correlated with the absolute arginase II intracellular concentration of the various neuroblastoma cell lines analyzed (SKN-MC > LAN-1 > Kelly; Supplementary Fig. S1D). Silencing of arginase II led to restoration of T-cell proliferation, confirming the specific role of arginase II (Supplementary Fig. S1G and S1H).

Neuroblastoma creates both a local and a systemic depletion in arginine, extending its immunosuppressive environment

Having established the ability of neuroblastoma cells to suppress T-cell proliferation in our model, we investigated the role of
arginase in human neuroblastoma samples. High arginase activity (mean, 1,100 mU/10⁶ cells) was confirmed in human GD2⁺ cells from patients' tumors at diagnosis, with rapid degradation of arginine into urea (Fig. 2A). Monocytes and neutrophils from healthy donors were used as controls. GD2⁺ cells expressed both arginase isoform genes (Fig. 2B). However, immunohistochemical examination of diagnostic tumor biopsy samples, confirmed the specific expression of arginase II within GD2⁺ tumor cells (Fig. 2C and Supplementary Fig. S2), with minimal staining for arginase I and iNOS enzymes. The findings are consistent with the established predominant expression of arginase II in healthy neuronal tissue (13). We performed T-cell proliferation assays using GD2⁺ tumor cells sorted from the neuroblastoma tumors. GD2⁺ cells showed a significant ability to suppress T-cell proliferation (Fig. 2D). Arginase inhibition with NOHA led to rescue of T-cell proliferation consistent with our earlier findings (10). No correlation was found between patient clinical characteristics and suppressive activity, including patient age, stage, or Myc-N status.

Patients with advanced neuroblastoma can present with systemic immune alterations, including lymphopenia (14, 15). It has previously been shown that arginase enzymes can be released by tumor cells to enhance the area of immunosuppression (10). We found no increase in arginase activity of culture supernatants (data not shown), indicating the enzyme was not released. However, patients with neuroblastoma frequently present with large tumor masses. We hypothesized that neuroblastoma may cause a systemic depletion of arginine due to consumption by the tumor mass, despite the failure to secrete free enzyme. Examination of plasma from patients with neuroblastoma at diagnosis showed a significant reduction in arginine concentrations in the blood, compared with healthy controls (Fig. 2E; healthy controls, 135 µmol/L vs. patients with neuroblastoma, 70 µmol/L; P = 0.0057). No increased plasma arginase activity was seen compared with the healthy controls, confirming the absence of secreted free enzyme (Supplementary Fig. S2b, P = 0.7139). To assess the effect of low arginine on T-cell proliferation, donor T cells were cultured in the plasma of patients with neuroblastoma or healthy donors. We showed that the low arginine in patient plasma significantly impaired T-cell proliferation compared with T cells cultured in plasma from healthy donors (Supplementary Fig. S2c, P = 0.045) and induces CD3ζ chain downregulation (Supplementary Fig. S2d). Consistent with previous findings exogenous arginine induces significant rescue of T-cell proliferation (P = 0.0038, Supplementary Fig. S2e). Thus,
our data suggest that neuroblastoma creates both a local and a systemic immunosuppressive microenvironment through the depletion of arginine by tumor cells.

The neuroblastoma microenvironment leads to altered immune cell frequency and function

To understand the impact of neuroblastoma on immune populations in vivo, we first characterized the role of neuroblastoma-derived arginase in a neuroblastoma murine model. TH-MYCEN mice are transgenic mice providing the closest biologic model of human neuroblastoma development (16). In patients, the most common location for neuroblastoma primary tumors is the adrenal gland. We compared the arginase activity of murine adrenals with murine neuroblastoma tumors and identified that tumor cells have a significantly higher arginase activity (Fig. 3A, \(P = 0.0004\)). Using a method described by Zhang and colleagues (17), extracellular fluid was isolated from neuroblastoma tumor tissue and found to have significantly lower arginine concentrations than healthy adrenal tissue (Fig. 3B, \(P < 0.0001\)). Similar to human tissue, GD2\(^+\) cells isolated from murine tumors predominantly expressed arginase II; however, low expression of arginase I is also found (Fig. 3C and Supplementary Fig. S3a). GD2\(^+\) cells suppress T-cell proliferation, which is restored by arginase inhibition. Data are representative of five independent experiments. Error bars, SD. E, plasma arginine is significantly lower in 22 newly diagnosed patients with neuroblastoma compared with 22 healthy donors.
mice. T-cell frequency in the spleens of tumor-bearing mice were significantly decreased (Supplementary Fig. S3b, bottom, $P = 0.03$), and T cells represented only a minor population of cells (3.2%) within the tumor mass (Fig. 4A). Myeloid cells are a second predominant immune population and have been shown to promote neuroblastoma chemoresistance (18). Within tumors, myeloid populations were composed of F4/80$^+$ macrophages (4.5%), Ly6C$^+$ (6.7%), and Ly6G$^+$ cells (2.7%; Fig. 4A). In our model, we identified that tumor-bearing mice also had increased numbers of F4/80$^+$ myeloid cells in the blood compared with controls (Supplementary Fig. S3, top; F4/80, $P = 0.0049$). In the spleen, all populations of myeloid cells were significantly increased compared with controls (Supplementary Fig. S3; F4/80, $P = 0.001$; Ly6G$^+$, $P = 0.0016$; Ly6C$^+$, $P = 0.04$).

It is recognized that myeloid cell activation plays a key role in coordinating the anticancer, proinflammatory immune response through cytokine release (19). To understand whether the neuroblastoma microenvironment affects the activation of myeloid cells, we isolated the 2 main populations of myeloid cells, Ly6C$^+$ or F4/80$^+$, from the spleen and tumors of tumor-bearing TH-MYCN mice and control mice, and tested their response to the potent immune stimulator LPS. Both myeloid populations had significantly impaired TNF$\alpha$ release compared with those from healthy controls (Fig. 4B and C), comparative to the effect of myeloid cells cultured in arginine-free conditions. No difference in IL10, IL12, or IL6 was identified suggesting an inactivation of myeloid function, as opposed to polarization (data not shown).
These murine studies identify the inhibitory role of the neuroblastoma arginase microenvironment on myeloid cell activation. To understand whether the arginine-deficient microenvironment in human neuroblastoma can also inactivate human myeloid cell function, CD14+ monocytes from healthy donors were cultured in the presence of neuroblastoma cell supernatant and stimulated overnight with LPS. Analogous to our murine study, neuroblastoma conditioned monocytes had decreased secretion of TNFα (Fig. 5A), and no increase in the T-cell activatory cytokine IL12 (Supplementary Fig. S4). Coculture with arginine-deficient media recapitulated these findings, confirming the specificity of the mechanism. Furthermore, no increase in the myeloid maturation marker CD86 was observed (Supplementary Fig. S4b). Similarly, no upregulation in the M2 polarization marker CD206 was seen, indicating that the monocytes are inactivated but not polarized (data not shown) by the low-arginine microenvironment of neuroblastoma.

It is well established that tumor-associated myeloid cells may support tumor pathogenesis by directly inhibiting T-cell function. Suppressive myeloid cells have been reported in neuroblastoma, although the mechanism of their induction and their relevance in patients has not been well-characterized (20). We co-cultured monocytes conditioned with arginine-deplete neuroblastoma media with T cells from healthy donors and found they are able to significantly suppress T-cell proliferation (Fig. 5B). As neuroblastoma creates a systemic arginine depletion, we also examined the myeloid populations in the blood of newly diagnosed patients (Supplementary Fig. S4c). An increase in circulating CD14+ (P = 0.034), but not CD15+ cells, was found compared with healthy controls (Fig. 5C, top and middle). These CD14+ monocytes from patients with neuroblastoma were capable of suppressing allo-}

**Clinical impact of neuroblastoma arginase microenvironment**

Our findings highlighted the key role of arginase activity by neuroblastoma tumors. To understand the clinical significance of our findings we interrogated the "R2: microarray analysis and visualization platform." Analysis of primary patient samples at diagnosis confirms the enzyme arginase II is expressed by all stages of neuroblastoma tumor, with the exception of stage 4S, at higher levels than arginase I (Supplementary Fig. S5a). High arginase II expression correlates with a significantly worse overall survival (Supplementary Fig. S5b). The result suggests a key role for arginase II in neuroblastoma pathogenesis. With regard to myeloid populations, patients with low intratumoral CD14 expression, suggestive of inactive myeloid cells, also do significantly worse (Supplementary Fig. S5c).

T-cell–based immunotherapies commonly require harvesting of T cells from patients for *ex vivo* manipulation (21). These studies assume that T cells from patients are fully functional, but our studies suggest that these cells would not be in an optimal condition. To test this concept, we isolated T cells from patients at diagnosis and found they have reduced proliferative capacity compared with those from healthy controls, suggesting they have been impaired by the immunosuppressive microenvironment created by the developing neuroblastoma (Fig. 6A). This observation complements our finding that T-cell frequency is also lower in patients with neuroblastoma than in healthy controls (P = 0.039; Fig. 5C, bottom). The cancer germline antigen NY-ESO-1 is
an antigenic target on neuroblastoma and has been suggested as a target for both endogenous immunity and novel T-cell therapies (22). To evaluate the impact of the arginase microenvironment on engineered T-cell immunity, NY-ESO-1-specific T-cell receptor (TCR)–engineered T cells were conditioned with neuroblastoma cell lines ex vivo. Neuroblastoma induced an arginase-dependent inhibition of proliferation by NY-ESO-1 TCR-engineered T cells, which was rescued by the arginase inhibition (Fig. 6B). Extending these findings, we tested the impact of the neuroblastoma microenvironment on engineered CAR T cells targeting GD2. We demonstrate that neuroblastoma leads to a significant impairment in cytotoxicity and proliferation of CAR T cells (Fig. 6C and Supplementary Fig. S4d), with some rescue in cytotoxicity when arginase is inhibited (Fig. 6D). These findings provide the first evidence for the role of neuroblastoma arginase activity in impairing antineuroblastoma T-cell therapies.

Patients with neuroblastoma, particularly those with advanced disease, can present with cytopenias. However, the mechanism by which neuroblastoma can impact on hematopoietic progenitors has never been reported. As neuroblastoma lowers plasma arginine, we hypothesized that the low-arginine microenvironment could also inhibit the proliferation of bone marrow hematopoietic progenitors. Human CD34+ hematopoietic stem cells (HSC) have significantly impaired cell division, with no loss of CD34+ expression, in the presence of neuroblastoma patient plasma (red histogram) compared with those cultured in plasma from healthy controls (blue histogram; Fig. 6E). CD34+ cells cocultured with tumor cells from patients or with arginine-deplete media had similar significant decreases in proliferation, compared with controls (Fig. 6F). Therefore, the arginase-dependent microenvironment of neuroblastoma not only suppresses T cells and...
myeloid cells but also inhibits the division and proliferation of CD34⁺ HSCs.

**Discussion**

In this study, we identify for the first time the ability of primary human neuroblastoma cells to directly inhibit T-cell proliferation through high arginase activity. We showed that GD2⁺ neuroblastoma cells from patients express predominantly the arginase II isoform. Arginase I and II are localized to the cytosol and mitochondria, respectively (23). In healthy states, arginase I is predominantly expressed by hepatocytes, whereas arginase II has wider, tissue-specific expression (24). Both isoforms catalyze the conversion of arginine into ornithine and urea. Neuroblastoma is a malignancy of the sympathetic nervous system, arising from neural crest progenitors that ordinarily develop into sympathetic ganglia and adrenal medulla (25, 26). Arginase II is the principal isoform expressed in the nervous system, with little or absent arginase I protein, consistent with our findings (27). Indeed arginine metabolism plays a key role in both healthy neuronal function and diseases of the nervous system and may contribute to the relative immunoprivileged niche that the nervous system exists in (28).

Using the "R2: microarray analysis and visualization platform," we confirmed that arginase II was expressed by all stages of neuroblastoma suggestive of a fundamental role in neuroblastoma pathogenesis (29). Stage 4s neuroblastoma is associated with an excellent prognosis, and it is interesting that in this subtype, arginase II gene expression is not higher than arginase I. Although the role of arginase I—expressing myeloid-derived suppressor cells (MDSC) in altering T-cell responses in patients with cancer has been well-established, arginase II in solid tumor immune biology has only received limited attention to date (30). We recently reported the role of arginase II in creating an immunosuppressive microenvironment in AML (10). Similarly, in prostate cancer, arginase II expression by both the tumor cells and cancer-
Associated fibroblasts serve to regulate immunosuppressive pathways (31). The increased expression and immunosuppressive potential of arginase II alone is likely to be tissue- and diseasespecific (32).

We examined the impact of neuroblastoma arginase activity to understand the effect on both local and systemic immunities. We first showed that neuroblastoma tumors lower local arginine concentrations to levels that inactivate surrounding T and myeloid cells. Increased arginase II expression correlates with decreased T and myeloid cell tumor infiltration in head and neck squamous cell carcinoma, supporting our findings in neuroblastoma (33). Arginine is exchanged between the blood and extracellular fluid to maintain homeostasis (34, 35). We investigated plasma levels of arginine in newly diagnosed patients with neuroblastoma and found them to be significantly lower than healthy controls. Similar changes were identified in mice with larger tumors. However, unlike AML, we show here that neuroblastoma does not release free arginine II enzyme. Therefore, uptake and catabolism by the tumor mass must presumably play a significant role in systemic depletion. Patients with high-stage neuroblastoma frequently present with significant cachexia (36). As tumor consumption of arginine lowers systemic arginine concentrations, physiologic compensation through protein breakdown and arginine recycling via the intestinal–renal axis may seek to maintain homeostasis (37). Corresponding decreases in plasma arginine have been described in patients with renal cell carcinoma and cervical cancer at diagnosis (38, 39).

Arginine is a semioessential amino acid required by healthy tissues for a number of cell processes, including cell viability, proliferation, and protein synthesis. Thus, arginase activity likely provides 2 key roles in neuroblastoma pathogenesis—first maintaining normal cell metabolism but second contributing to immune escape (40, 41). It is established that arginine depletion leads to T cell-cycle arrest, impaired proliferation, and reduced activation (42, 43). This is consistent with our findings of both lower T-cell numbers in the peripheral blood and their impaired proliferative capacity. The "R2: microarray analysis and visualization platform" identifies that neuroblastoma tumors with low CD3 expression have a worse overall survival, supporting the pathogenic role of arginine depletion in neuroblastoma tumors. Monocytes and macrophages play a key role in antitumor immunity through coordination of other immune effectors by cytokine secretion. Consistent with previous findings, we identify that CD14+ myeloid cells are present within the tumor microenvironment (20, 44). However, we describe for the first time that neuroblastoma arginase activity impairs the ability of monocytes to respond to an inflammatory stimulus and decreases their production of the immune activatory cytokine TNFα from myeloid cells. A previous study noted an impaired TNFα-driven dendritic cell stimulation of T cells in neuroblastoma but no mechanism was found (9).

In both our murine model and in patients' blood, we observed a significant increase in the number of immunosuppressive monocytes. It has been shown in xenografts that immunosuppressive myeloid cells are associated with neuroblastoma but the mechanism of cross-talk has not been identified (45). Here, we identify that neuroblastoma arginase activity plays a key role in modulating the CD14 immune phenotype. The clinical finding of cytopenias in patients with neuroblastoma is also well-recognized. However, the mechanism of neuroblastoma-induced cytopenia is unclear, particularly in metastatic or end-stage disease where the bone marrow is rarely significantly replaced by tumor cells. We show for the first time that the low-arginine microenvironment of neuroblastoma suppresses CD34+ hematopoietic cell division and proliferation.

Immunotherapy requiring an active innate and adaptive immune response has become a major new approach in the treatment of high-risk neuroblastoma. Despite a promising preclinical rationale, immunotherapy clinical trials in patients with neuroblastoma have not resulted in significant improvements in overall survival (6). Studies show an unexplained decrease in adoptive T-cell numbers after administration, with no correlation between the dose of engineered cells administered, their numbers in peripheral blood or antitumor response (5). In xenograft studies of neuroblastoma cell vaccines, T cells from tumor-bearing mice have defective antitumor immune responses, both locally at the site of injection and systemically, although no mechanism has been identified (46). Furthermore, immunotherapies that have activity alone in vitro often require co-administration with cytokines to maximize therapeutic effect in patients. Early-phase clinical trials using autologous patient-derived dendritic cell vaccines illustrate that moderate benefit is only seen when co-administered with IL2 (47).

NY-ESO-1 is a cancer germline antigen known to be highly immunogenic in patients with melanoma and also is expressed by the majority of neuroblastomas. Although it has been demonstrated through ex vivo manipulation that patients can generate antitumor immune responses, both locally at the site of injection and systemically, although no mechanism has been identified (46). Furthermore, immunotherapies that have activity alone in vitro often require co-administration with cytokines to maximize therapeutic effect in patients. Early-phase clinical trials using autologous patient-derived dendritic cell vaccines illustrate that moderate benefit is only seen when co-administered with IL2 (47).

Following the identification of neuroblastoma surface ganglioside D2 as a potential therapeutic target, CAR-engineered T cells are an alternative therapeutic approach under development. However, similar to vaccine approaches, early-phase trial results have not matched preclinical results. We find that the arginase-dependent microenvironment created by neuroblastoma can significantly inhibit the proliferation and cytotoxicity of anti-GD2 CAR T cells. This is the first report identifying a mechanism in which CAR T-cell activity can be impaired by tumor immuno-suppression. Interestingly, a previous report assessing CAR-engineered T-cell responses against a neuroblastoma cell line in vitro identified a similar decrease in activity but no mechanism for this was identified (49). Most early trials of novel immune therapies enroll refractory or relapsed patients who may have very significant disease burden, due to treatment failure. It is therefore unsurprising that the extent of the immunosuppressive microenvironment both within the tumor mass and blood has the ability to hamper adaptive T-cell responses.

Arginine supplementation has been administered to patients, in nontumor settings, to enhance immunity and tissue repair. In a subcutaneous xenograft model of neuroblastoma supplemented with combination arginine and IL2, lymphokine-activated killer cells demonstrated increased antineuroblastoma immunity and mice had prolonged survival (50). However, concerns that arginine supplementation may feed tumor growth would need to be evaluated further. Therapeutic targeting through arginase inhibition is a more likely strategy. Although small molecules such as NOHA have been shown to be toxic in vivo, other compounds that...
act on arginase are already under preclinical evaluation. A recent study identified that the green tea derivative polyphenon E can promote antitumor immunity in a murine model of neuroblastoma. Polyphenols have been shown to have activity against arginase. It is possible that the activity previously reported may have been also due to blockade of arginase expressed by neuroblastoma cells.

In conclusion, our findings provide evidence for the key role of arginase activity in the creation of an immunosuppressive microenvironment in neuroblastoma and have significant clinical implications for T-cell immunotherapy approaches. Tumor escape from different arms of the immune response is likely to be multifactorial, and control of arginase activity by tumor tissue fits rationally with other mechanisms identified in murine models (48). Targeting of arginase activity in neuroblastoma could provide a new way of enhancing both autologous and therapeutic antineuroblastoma immunity.

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

Authors’ Contributions
Conception and design: F. Mussai, K. Petrie, J. Anderson, L. Chesler, C. De Santo
Development of methodology: F. Mussai, S. Egan, R. Wheat, L. Chesler, C. De Santo

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