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IL-1ß and TNF-α create an immune-metabolic loop regulating Arginase 2 via p38-ERK in neuroblastoma

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Summary

Neuroblastoma is the most common solid tumour of childhood, yet the prognosis for high risk disease remains poor. We demonstrate that arginine metabolism via Arginase 2 (ARG2) drives neuroblastoma cell proliferation. Proteomic analysis reveals Stage IV human tumours have a Th1-skewed intra-tumoural microenvironment and tumour cell arginase infiltrating-monocytes to a CD68+ phenotype, which release IL-1α and TNF-β in a AKT dependent manner. Il-1β and TNF-α signal-back to upregulate ARG2 expression via p38 and ERK1/ signalling in neuroblastoma and neural crest. Tumour cells express ARG2 mis-localised in the cell cytoplasm, whilst deficiencies in Argininosuccinate Synthase (ASS) and Ornithine Transcarbamylase (OTC) enzymes result in tumour cells reliant on extracellular arginine. Therapeutic depletion of arginine with PEGylated-recombinant Arginase is cytotoxic to neuroblastoma and leads to a significant extension in transgenic murine survival. Thus we describe an immune-metabolic regulatory loop between tumour cells and infiltrating myeloid cells regulating ARG2, which can be clinically exploited.

Introduction

The consumption and metabolism of diverse nutrients by cancer cells is recognised as a key regulator of immunity. Glucose metabolism by cancer cells generates a tumour microenvironment that has low levels of glucose, leading to inhibition of T cell cytotoxicity through the accumulation of lactate, microenvironment acidification, and reduced aerobic glycolysis (Angelin et al., 2017, Calcinotto et al., 2012, Ho et al., 2015). Tumour infiltrating monocyte differentiation and cytokine release may be similarly affected, leading to perturbation of their role in coordinating the surrounding immune response (Dietl et al., 2010, Wei et al., 2006). Amino acid metabolism also plays a critical role in the function of both normal and malignant cells. Although whole body amino acid homeostasis is regulated through restricted inter-organ enzyme expression, at the cellular level enzyme expression is controlled in the intracellular compartment to maintain metabolic precursor supplies and regulate the wider tissue microenvironment (Fultang et al., 2016).

Arginine is a semi-essential amino acid, that is metabolised into ornithine and urea by the expression of cytoplasmic Arginase 1 and mitochondrial Arginase 2 (ARG2), or Nitric Oxide Synthase (NOS) enzymes into reactive nitric oxide species (Morris, 2016). These metabolites feed forward into diverse roles in cell signalling, proliferation and protein synthesis. Cellular breakdown of arginine also plays a critical role in regulating the immune response, a process which has been capitalised on by malignant cells to contribute to their immune escape (De Santo et al., 2010). We recently identified that Acute Myeloid Leukaemias (AML) and neuroblastoma, two of the most common and devastating cancers of childhood create a

potent immunosuppressive microenvironment through the expression of ARG2 enzyme which suppresses T-cell immunity (Mussai et al., 2015b, Mussai et al., 2013).

Although the metabolic effect of cancer cells on shaping the responsiveness of surrounding immune populations is increasingly well described, the reciprocal effects of immune cell populations on modulating cancer cell amino acid metabolism have not previously been reported. In particular the role of arginine metabolism in this process is unknown and the signals which regulate ARG2 in cancer are unknown. Here we demonstrate how myeloid cells within the tumour microenvironment and tumour cells engage in reciprocal cross-talk to regulate the expression of ARG2 in neuroblastoma cells, and how this arginine metabolism plays a central role in neuroblastoma pathogenesis. Importantly the study identifies arginine metabolism as a clinically relevant therapeutic target.

Results

Stage IV neuroblastomas have a Th1 cytokine enriched microenvironment

We established that ARG2 expression by neuroblastoma cells creates an immunosuppressive microenvironment and contributes to escape from patients' immune responses and immunotherapy (Mussai et al., 2015b). However, the role of ARG2 in neuroblastoma development and more widely in human cancers has only received limited study. Expression of ARG2 in human GD2+ neuroblastoma cells was confirmed by immunohistochemistry of tumour biopsies at diagnosis (Figure S1A). To understand if arginine metabolism is central to tumour cell growth we first blocked uptake of arginine from the microenvironment via Cationic Amino Acid Transporter-1 (CAT1). N-nitro-L-arginine (L-NAME) led to a significant decrease in tumour cell proliferation (Figure 1A). Culture of tumour cells in the absence of arginine similarly inhibited tumour cell metabolic activity (Figure 1B). shRNA knock-down of ARG2 led to a significant reduction in cell proliferation in the highest expressing cells (SKNMC>KELLY) (Figure 1C, and Figure S1B). Thus arginine metabolism by ARG2 in neuroblastoma cells contributes to tumour cell proliferation.

The pathways which regulate ARG2 expression in cancer cells have not been reported to date. We showed that ARG2 expression is highest in Stage IV tumours and is associated with a worse overall survival (Mussai et al., 2015b). To gain a deeper understanding of the immune microenvironment within these tumours, we investigated the proteomic profile inside 23 human neuroblastomas at diagnosis (9 Stage I and 14 Stage IV) using a novel antibody proteomic platform that characterises the expression of 900-cancer-related proteins (Ruiz-Babot et al., 2018). Non-metric multidimensional scaling for all analysed samples based on

the complete protein expression data revealed separate clustering of Stage I and Stage IV tumours (Figure 1D). Heat-map representation of protein signals reveal that Stage I and Stage IV tumours show distinct molecular proteomic subgroups, with 7 Stage IV tumours (P21-27) forming a distinct group, while 3 others (P10,P15,P16), had proteomes closer to Stage I tumours (Figure S1C; Related to Figure 1D). Characterisation of the Stage IV tumours identified significantly higher levels of the Th1 cytokines IL-1β, IFN-γ and TNF-α than Stage I tumours (Figure 1E). In contrast, Stage I tumours had increased expression of the Th2-related cytokines TGF-β, IL-10, and IL-4 (Figure 1F). No significant differences in IL-6 and IL-13 expression were identified. The finding of high levels of IL-1β, IFN-γ and TNF-α identifies that Stage IV tumours are skewed towards a Th1 microenvironment. Tissue micro-arrays of 27 human neuroblastomas at diagnosis validated the expression of IL-1β, TNF-α and IFN-γ within tumour samples compared to healthy peripheral nerve tissue (Figure 1G). No association of cytokine expression with anatomical tumour site was identified.

We hypothesised the cytokine microenvironment may help drive tumour development. Cytokines may be functional either within the tumour microenvironment or released into the blood to induce systemic effects. Analysis of blood from 25 neuroblastoma patients at diagnosis revealed that the majority of patients did not have significantly increased TNF-α and IFN-γ compared to healthy controls, although in 9 cases circulating levels of IL-1β and IL-6 were significantly higher (p=0.042) (Figure 1H, Figure S2A). Thus Th1 cytokines are predominantly localised to the intra-tumoral microenvironment to exert their effects. Consistent with this, high expression of IL-1β or TNF-α within tumours is associated with a significantly worse overall survival for neuroblastoma patients (n=88, p=0.012 and p=0.027

respectively (Figures S2B and S2C). In contrast high IFN-γ is associated with a significantly better prognosis for patients (p=0.0036) suggesting these cytokines may have differential effects on tumour cells (Figure S2D). We demonstrated that primary human tumour cells are positive for IL1R1, TNFRSF1A (TNFR1), and IFNGR1 mRNA expression (which are receptors for IL-1β, TNF-α and IFN-γ respectively), although only IL1R and TNFR1 proteins are expressed on the cell surface (Figure 1I and Figure S3A). Therefore neuroblastoma cells have the capacity to respond to cytokines within the tumour microenvironment.

Tumour-conditioned monocytes cells release IL-1β and TNF-α

As tumour cells released minimal cytokines (<8pg/ml, Figure S3B) we hypothesised that myeloid cells within the tumour may be releasing IL-1β and TNF-α. Myeloid cells are a major population inside tumours which can shape the surrounding immune response through cytokine release (Engblom et al., 2016). We have previously shown that myeloid cells are increased in the blood of patients with neuroblastoma and correlate with a worse overall survival (Mussai et al., 2015b). Immunohistochemistry of tissue microarrays of 27 tumours identified expression of IL-1β and TNF-α in infiltrating myeloid cells. (Figure 2A; Figure S3C). Analysis of human neuroblastoma proteomes showed increased expression for the monocytic cell marker CD14 and the granulocytic cell marker CD15 in Stage IV vs Stage I tumours (Figure S4A). Whilst CD15 staining localised around epithelia (Figure S4B), CD14+ monocytes and CD68+ macrophages infiltrated the tumour tissue (Figure 2B and Figure 2C).

We hypothesised that tumour cell catabolism of arginine in the microenvironment could modulate myeloid function. Culture of CD14+ cells with sorted-GD2+ expressing neuroblastoma cells from patients or cell lines led to upregulation of the macrophage marker CD68 (Figure 2D and Figure S4C), which was prevented by inhibition of tumour arginase activity with L-NOHA. A minority of cells upregulated the M2 marker CD206 (Figure S4D). Myeloid Arginase I activity was down-regulated (Figure S4E) consistent with a M1-like phenotype. Culture of CD14+ cells in low arginine media upregulated CD68, confirming our findings (Figure S4F).

Next we investigated if neuroblastoma conditioned monocytes could be responsible for the IL-1β and TNF-α in the tumour microenvironment. Tumour conditioning led to an increased frequency of IL-1β and TNF-α positive cells at 24hours (Figure S5A) and 48hours (Figure 2E) and release of IL-1β and TNF-α into the culture supernatants (Figure 2F). Similarly CD14+ cells sorted from neuroblastoma patients expressed IL-1β and TNF-α (Figure S4B). IL-1β and TNF-α secretion from myeloid cells is regulated by AKT signalling (Xie et al., 2014). Neuroblastoma cell co-culture led to AKT phosphorylation in CD14+ cells (Figure 2G) and AKT inhibition with MK-2206 prevented IL-1β and TNF-α release (Figure 2H). Although CD15+ granulocytes released IL-8, they did not release either IL-1β or TNF-α following tumour co-culture (Figure S5C). Therefore arginine metabolism by neuroblastoma cells polarises surrounding monocytes to upregulate CD68 and release IL-1β and TNF-α.

Il-1β and TNF-α drive ARG2 expression in a p38/ERK signalling

The regulation of ARG2 by the immune response has not been explored. As ARG2 contributes to tumour cell proliferation, we hypothesised that these cytokines may reciprocally regulate

ARG2 expression. We first showed that Th1 (IL-1β, TNF-α, IFN-γ) but not Th2 (TGF-β, IL-10, and IL-4) cytokines upregulated ARG2 expression (Figure S5D). Consistent with this finding, supernatant from the tumour conditioned myeloid cells upregulated ARG2 in the neuroblastoma cell (Figure 3A, Figure S5E). Treatment of neuroblastoma cells with low basal expression of ARG2 (SKNAS and IMR32) with IL-1β and TNF-α, either alone or in combination, resulted in upregulated ARG2 expression (Figure 3B). IFNγ had no effect on ARG2 expression (Figure S5F). Treatment of sorted human GD2+ neuroblastoma cells similarly upregulated ARG2 (Figure 3C).

Neuroblastoma is a pathological derivative of neural crest cells, which usually develop into diverse populations including dorsal root ganglia, melanocytes, odontoblasts, and schwann cells. We hypothesised that an inflammatory microenvironment containing similar factors to postnatal inflammation may contribute to tumour initiation by upregulating ARG2 in these embryological cells. Transcriptomic analysis indicates that ARG2 is the main arginase isoform expressed by human neural crest cells (n=5, R2: array) (Figure S5G), although ARG2 expression is significantly lower than in tumour cells (Figure 3D). Analogous to neuroblastoma, treating cultures of multipotent human embryonic dorsal root ganglion precursors with IL-1β and TNF-α led to a significant upregulation of ARG2 protein expression (Figure 3E), demonstrating the inherent responsiveness of neural progenitors to these signals before oncogenic transformation.

The IL1R1 and TNFR1 receptors can induce a signalling cascade that both converge on a common final effector pathway through ERK1/2 and p38 activation, and MSK1 activation (Figure 3F) (Turner et al., 2014, Campbell et al., 2004, Qin et al., 2004). Consistently, treatment

of neuroblastoma cells with IL-1β and TNF-α leads to NFκB phosphorylation by 0.5hours and subsequent phosphorylation of ERK1/2 from 1 hour onwards (Figure 3G). Simultaneously, the cytokines also induced p38 phosphorylation at 0.5 hours (Figure 3G). PD98059 binds inactive ERK and prevents phosphorylation and activation by upstream mediators, whilst SB20308 inhibits p38 catalytic activity but does not affect phosphorylation. The resulting inhibition of p38 or ERK1/2 signalling leads to subsequent downregulation of ARG2 expression in SKNAS neuroblastoma cells (Figure 3H). MSK1 is at crossroads of the common downstream cascade and can be auto-regulated by kinases including ERK1/2 and p38. SB747651A blockade of MSK1 activity, which is phosphorylated from 0.5 hours onwards (Figure 3G), similarly prevented cytokine induced ARG2 upregulation (Figure 3I). ARG2 in neuroblastoma is thus upregulated by IL-1β and TNF-α within the tumour microenvironment in a p38 and ERK1/2 dependent manner.

Neuroblastoma cells have a defective arginine recycling pathway

The identification of ARG2 regulation by cytokines prompted a deeper analysis of the state of arginine metabolism in neuroblastoma cells and neural crest cells. ARG2 is classically described as a mitochondrial enzyme which leads to the catabolism of arginine into ornithine for downstream cell processes. We hypothesised that IL-1β and TNF-α may also alter trafficking of ARG2 as part of the malignant process. Confocal microscopy of embryonic neural crest-derived ganglion cells showed that ARG2 co-localised in the mitochondria, with no change in localisation after exposure to cytokines (Figure S6A). However, Western blots of fractionated neuroblastoma cells confirmed the presence of ARG2 in both cell cytoplasm and

mitochondria (Figure 4A), with the enzymes in both compartments metabolically active in catabolising arginine to ornithine and urea (Figure 4B). The finding of intracellular ARG2 outside the mitochondria in cancer cells has not been previously reported. Subcellular ARG2 trafficking is dependent on cleavage of the mitochondrial targeting sequence (MTS) of ARG2 by Mitochondrial Protein Peptidase (MPP) localised in the mitochondria (Pandey et al., 2014). Analysis of neuroblastoma cells shows that MPP is found in the cytoplasm (Figure 4A) where cleavage of the MTS may trap some of the enzyme in the cytoplasm. Confocal microscopy confirmed that although the cytokines increased total ARG2 no further changes in cytoplasmic or mitochondrial distribution were seen (Figure 4C).

One hallmark of an inflammatory tumour microenvironment is the release of the acute phase protein serum amyloid A (SAA) (De Santo et al., 2010, Ye and Sun, 2015). In vascular cell lines the SAA-oxidised low density lipoprotein (oxLDL)-OLR1 signalling cascade has also been reported to modulate intracellular trafficking of ARG2 (Witting et al., 2011). Analysis of neuroblastoma patients at diagnosis identified high levels of intra-tumoral and circulating SAA (Figure 4D and Figure 4E). Expression of SAA receptors (Figure 4F) and the oxLDL receptor OLR1 (Figure S6B) were confirmed on tumour cells. However treatment of neuroblastoma cells with SAA and oxLDL did not alter ARG2 localisation (Figure 4G and Figure S6C) or alter its expression (Figure 4H), confirming these factors play no role in the regulation of neuroblastoma ARG2.

Cells consume arginine from the extracellular space through the expression of transmembrane transporters belonging to the CAT family of proteins (Fultang et al., 2016). Once inside the cell arginine is catabolised to ornithine and urea through the expression of ARG1 and ARG2 enzymes or to nitric oxide species through the expression of NOS family of proteins (Figure 5A). Neuroblastoma expresses ARG2 that converts arginine into ornithine and urea. Arginine can then be resynthesized through the conversion of ornithine to citrulline by OTC, and then citrulline to argininosuccinate by ASS1. Finally argininosuccinate is converted back to arginine by arginine succinate lyase (ASL) which is constitutively expressed in all normal and malignant cells. The pathway plays a key role in allowing healthy cells to tolerate conditions when arginine concentrations in the microenvironment are low. RT-qPCR of GD2+ cells isolated from bone marrows of 10 neuroblastoma patients identified relatively high ASL expression but low ASS and OTC expression. (Figure 5B). Western blotting analysis shows neural crest-derived primary cells and neuroblastoma cell lines have variable expression of ASS and OTC (Figure 5C), which we reconfirmed by immunohistochemistry of patient tumour samples (Figure 5D). Transcriptomic profiling of 88 tumour samples confirmed the presence of ASS and OTC mRNA (Figure S7A). To investigate if IL-1β and TNF-α could modulate the expression of arginine re-synthesis enzymes, neuroblastoma cells were placed in culture with these cytokines. No significant change in ASS, OTC, or CAT1 mRNA expression levels was identified (Figure 5E). The absence of a complete cycle of arginine pathway enzymes indicates that neuroblastoma cells are auxotrophic for arginine.

Neuroblastoma arginine auxotrophism can be therapeutically exploited.

The failure of cancer cells to recycle arginine makes them reliant on extracellular arginine. One approach to target arginine metabolism is therefore to block arginine uptake through inhibition of CAT1. Although this approach can be applied *in vitro*, CAT1 or arginase inhibitors are not yet established in the clinical setting due to issues over bioavailability *in vivo*. Instead the most clinically advanced approach is to completely deplete systemic arginine levels thus removing the substrate availability for metabolism.

BCT-100 is a PEGylated recombinant human arginase that we have shown can deplete arginine to undetectable levels in cancer patients leading to clinical responses in adult trials (Yau et al., 2015). Small reductions in arginine concentrations in the microenvironment may lead to compensatory upregulation of arginine recycling enzymes. As neuroblastoma cells exhibit variable ASS and OTC expression at baseline we investigated the effect of therapeutic arginine starvation on these enzymes. BCT-100 led to significant upregulation of ASS, OTC and CAT-1 in the majority of samples (Figure S7B). However culture of neuroblastoma cells with BCT-100 led to a rapid inhibition of cell proliferation (Figure 5F), and upregulation of cyclins D1 and A1 confirming cell cycle arrest (Figure 5G). Nutrient depletion can lead to quiescence or cell death (Philip et al., 2003). Consistent with this, culture of neuroblastoma cells with BCT-100 ultimately results in tumour cell death (Figure 5H) characterised by PARP cleavage (Figure 5I), but no increase in autophagic LC3II turnover (Figure S7C). Electron microscopy of sorted tumour cells from patients confirms loss of cell membrane integrity, and cellular fragmentation (Figure 5J).

These findings demonstrate that arginine deprivation could have clinical efficacy against neuroblastoma. To investigate this further we used the gold-standard *TH-MYCN* transgenic mouse model of neuroblastoma, in which the tyrosine hydroxylase promoter drives human MYCN expression and spontaneously develop tumours akin to high-risk *MYC*-amplified human neuroblastoma (Weiss et al., 1997). We first demonstrated that *ex vivo* treatment of murine GD2+ tumour cells with BCT-100 led to a significant reduction in viable cells (Figure 6A). Consistent with our findings in human neuroblastoma cells described above, murine tumour cells also expressed ARG2 (Figure S7D). Immunohistochemical staining of murine tumours showed variable expression of ASS and OTC (Figure 6B).

Next we investigated the effect of BCT-100 on tumour growth in 2 settings. Treatment of *TH-MYCN* with twice-weekly BCT-100 led to a sustained drop in plasma arginine levels to almost undetectable levels (Figure 6C). To investigate if therapeutic arginine deprivation could induce secondary effects on the frequency of key immune populations we analysed composition in the spleen, blood, and tumours after treatment. No difference in myeloid cells (monocytes, macrophages, granulocytes) and T cells was seen after the BCT-100 treatment (Figure 6D – F).

To understand if tumour growth could be delayed or prevented, mice were treated prophylactically from the time of weaning at 3 weeks of age, when the tumours were 1-2mm in size. Neuroblastoma development was significantly delayed and mice survived for significantly longer in the BCT-100 treated group compared to control ($p=0.0001$) (Figure 7A. Following this we investigated the effect of BCT-100 on established tumours. Here we showed that murine tumour progression was significantly delayed compared to the saline control and

overall survival was significantly extended from 5 to 10 days from the start of treatment (p=0.0181) (Figure 7B). No evidence for BCT-100 drug toxicity was identified.

To examine the global effects of arginine deprivation on neuroblastoma *in vivo*, tumours from control and BCT-100 treated mice were subjected to transcriptomic analysis. Arginase enzyme expression was downregulated, with upregulation up CAT1 (encoded by *SLC7A1*) consistent with the above findings in cell lines (Figure 7C). Treatment of tumour-bearing mice with BCT-100 also led to downregulation of ARG2. We validated this findings by western blotting (Figure 7D). The top 50 genes with the most significant differential in gene expression are shown in Figure 7E. BCT-100 led to an upregulation of the CLOCK Interacting Pacemaker (CIPC). CIPC is a regulator of transcriptional activity in the circadian-clock mechanism. MYC expression has been shown to modulate glucose metabolism in neuroblastoma contributing to malignant transformation (Altman et al., 2015). A number of cell matrix genes were also upregulated – Tenascin (Tnxb), Elastin (Eln), and Adhesion Molecule with Ig like domain 1 (AMIGO1).

In summary the dependence of neuroblastoma on arginine metabolism can be therapeutically targeted using PEG-recombinant Arginase (BCT-100), leading to neuroblastoma cell death and a significant prolongation in murine overall survival. This data provides a strong rationale for clinical translation.

Discussion

In this study we identify a novel a key reciprocal regulation between tumour arginine metabolism and intra-tumoral immunity. We demonstrated that the proteome of Stage IV tumours is markedly different from those of Stage I tumours, with notable expression of the pro-inflammatory cytokines IL-1β and TNF-α. These cytokines are released by CD14+ M1-like monocytes polarised by the low arginine tumour microenvironment in an AKT dependent manner. The cytokines in turn signal back to neuroblastoma cells to upregulate ARG2 expression in an ERK1/2-p38 dependent fashion, the first signalling pathway reported to control ARG2. The central role of arginine in neuroblastoma pathogenesis is compounded by the absence of ASS and OTC enzymes in neuroblastoma, confirming that tumour cells are reliant on extracellular sources of arginine. We show that therapeutic deprivation of arginine with the PEG-recombinant arginase BCT-100 leads to neuroblastoma cell death and prolonged survival of tumour-bearing mice, providing an exciting new therapeutic approach which can be rapidly translated for the treatment of this devastating childhood tumour.

Clinically it has long been clear that Stage I and Stage IV neuroblastomas are distinct at the levels of tumour dissemination, responses to chemotherapy, and patient outcome. Inter-cellular signalling within tumours remains difficult to characterise, although much has been learned from transcriptomic and epigenetic profiling of these tumours (Henrich et al., 2016, van Groningen et al., 2017). The functional interaction of proteins within cells and the cellular ecosystem must be contributing to variation in tumour aggressiveness, although analysis of multiple proteins inside tumours is challenging. To our knowledge, this is the first proteomic

characterisation of primary human neuroblastomas and the findings suggest that array-based proteomic profiling can lead to new insights into tumour biology.

Neural crest cells are highly multipotent stem cells in the embryo which give rise to a number of cell types such as melanocytes, odontoblasts, Schwann cells, peripheral neurons and specific endocrine cells including the adrenal medulla. We identified that neural crest cells are enriched in ARG2 protein, relative to ARG1. The role of ARG2 in embryological processes is not well understood. Neonatal CD71+ erythroid cells express ARG2 which may affect response to commensal bacteria in the developing baby, while dendritic cells in the developing foetus similarly express ARG2 to modulate immune responses *in utero* (Elahi et al., 2013, McGovern et al., 2017). That expression of ARG2 in neural crest-derived untransformed stem cells can be upregulated by the immune cytokines IL-1β and TNF-α, also points to the potential for metabolic change that can occur during malignant transformation or expansion. We have previously shown that AML blasts have similarly upregulated ARG2 in comparison to their non-malignant haematopoietic counterparts (Mussai et al., 2013). Indeed knock down of ARG2 significantly reduces the ability of tumour cells to proliferate in both cell types, suggesting this enzyme provides an advantage to cancer dissemination.

The regulators of ARG2 expression in cancer are poorly understood, despite abundant data on its cytoplasmic counterpart ARG1. ARG2 can be upregulated by LPS in murine intestinal epithelial cells or by hypoxia in pulmonary artery smooth muscle cells (Talavera et al., 2017, Xue et al., 2017). In cancer, hypoxia may also upregulate ARG2 in osteosarcoma cells, while obesity is correlated with increased ARG2 levels in pancreatic ductal adenocarcinoma models,

enhancing tumour growth (Setty et al., 2017, Zaytouni et al., 2017). We showed that Stage IV tumours have a predominance for cytokines usually described as pro-inflammatory or 'Th1', whilst the converse is true for Stage I tumours. To date the role of IL-1β and TNF-α in neuroblastoma has primarily centred around the effects of these cytokines on neuroblastoma cell lines used as models of neurodegenerative disease, such as Alzheimer's disease. In terms of its effects on the malignant phenotype, recombinant TNF-α has been shown to be a growth factor for neuroblastoma cell lines, although the mechanism of action was unknown (Goillot et al., 1992). A minor subset of neuroblastoma cells within tumours may themselves express TNF-α intracellularly or on the cell membrane, but they do not release the cytokine into the microenvironment (Liu et al., 2012). For IL-1β little is known in the context of neuroblastoma although it is reported to drive cyclo-oxygenase (COX-2) expression in neuroblastoma Alzheimer's disease cell line models (Fiebich et al., 2000). Importantly, we identified that levels of IL-1β and TNF-α proteins in the plasma are not significantly greater than in healthy donors, indicating that it is the intra-tumoral interactions that are key.

We and others have previously reported the ability of neuroblastoma cells to modulate surrounding monocytes into an immunosuppressive phenotype on T cells and NKT cells, through arginase activity (Liu et al., 2012). Here, we demonstrate that the depletion of arginine by tumour cell ARG2 polarises monocytic cells to a M1-like phenotype, with expression and release of IL-1β and TNF-α under the control of AKT. AKT inhibitors such as Perifosine have recently undergone early phase clinical trial development, including evaluation in refractory neuroblastoma, which suggests that targeting this pathway could prolong progression-free survival (Kushner et al., 2017). We show that that tumour-polarised

myeloid cells act in a paracrine and reciprocal fashion to regulate cancer cell metabolism through cytokine release. ARG2 expression is under the control of both p38 and ERK1/2 in neuroblastoma cells, and lies downstream of the receptors for IL-1β and TNF-α. Some redundancy in the signalling cascade is evident, such that inhibition of both pathways or their common effect on MSK1 are required to inhibit enzyme expression. The role of these pathways in cancer cell expression of ARG2 has not previously been reported. We have shown that neuroblastoma cells and surrounding myeloid cells cross-regulate in a feedback loop of metabolic and immune signalling.

It has been hypothesised that an immune-stimulatory event, such as infection in early childhood could contribute to the development of childhood cancers either through a normal or aberrant response. Indeed a 'delayed infection' hypothesis had been suggested for childhood acute lymphoblastic leukaemia (Greaves, 2006). Although specific infections like Epstein-Barr Virus are directly linked to malignant transformation of cells in Hodgkin's Lymphoma or nasopharyngeal carcinoma, in the majority of paediatric malignancies no evidence of clearly defined cause and effect have been found (Schmidt et al., 2010, Hwee et al., 2018). Our findings suggest that an isolated pro-inflammatory response within a tissue microenvironment could potentially lead to a cytokine profile that drives metabolism in malignant or pre-malignant cells, giving them a survival advantage, and allowing development into a frank malignancy. As to whether the inflammation is secondary to very specific infectious agents or is an abnormal, pathological response due to immune defects is difficult to define.

Although arginine metabolism under cytokine control can drive neuroblastoma proliferation, this axis also provides a potential therapeutic target. Targeting tumour-associated myeloid cells has received significant attention to date. Although depletions of myeloid cells can be achieved *in vivo* using anti-GR1 or anti-CR2 antibodies the effects are very short lived and no human equivalent exists for clinical translation. One approach to target the feedback loop we have described is to inhibit Il-1β and TNF-α cytokine activity. Anti-TNF-α therapy was the paradigm for anti-cytokine therapies with the development of anti-TNF-α antibody (infliximab) and a decoy anti-TNF-A receptor (etanercept). Although these antibodies have demonstrated remarkable activity in autoimmune conditions, they have only undergone limited study in the setting of cancer therapy. Infliximab has been used as a single agent in patients with advanced cancer with some patients experiencing disease stabilisation (Brown et al., 2008). The drug has also been trialled against renal cell carcinoma and although improvements in immune profiles were noted, there were also significant increases in adverse events (Larkin et al., 2010, Harrison et al., 2007). Similar antibodies against IL-1β (canakinumab) and its receptor IL-1R1 (anakinra) also exist. Although canakinumab has not been formally tested in patients with an existing cancer, administration of this drug has been shown to significantly reduce incidences of lung cancer and its mortality in patients with atherosclerosis (Ridker et al., 2017). Future combination clinical trials of these agents could represent a novel and potential approach in children with neuroblastoma.

We demonstrate that neuroblastomas aberrantly express ARG2 in the cell cytoplasm and that they have variable expression of the arginine recycling enzymes ASS and OTC. The result is

that neuroblastomas consume arginine from the microenvironment and are thereby susceptible to conditions of arginine starvation. We have shown here that it is possible to successfully target this pathway through therapeutic arginine depletion with BCT-100, a PEGylated recombinant arginase that induces a sustained arginine depletion for months in human trials (Mussai et al., 2015a, Yau et al., 2013). The drug has completed Phase I/II trials in adult malignancies with an excellent safety profile (Yau et al., 2015). Here we demonstrated that BCT-100 not only leads to a decrease in neuroblastoma proliferation followed by cell death *in vitro*, but also to delayed progression and prolonged survival in neuroblastoma-bearing mice. These findings support the testing of BCT-100 in an international Phase I/II clinical trial (PARC, NCT03455140) in children with relapsed/refractory malignancies including neuroblastoma. The targeting of both immune and metabolic drivers of tumorigenesis is as presented in this study, rational and clinically achievable, and could be a new paradigm in the treatment of neuroblastoma.

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Author Contributions

Contribution: F.M. and C.D.S designed the study, performed research, analysed data and wrote the manuscript. F.M. additionally secured ethical approval and was chief investigator of the study. L.F designed and performed research, L.G., L.G., A.B., S.E., F.B., O.Y., S.Br., A.V. J.M., G.E. performed research. M.N., M.H., SAB, L.C. D.Z. designed and supervised research. R.S. designed and performed research. C.M. provided neuroblastomas and P.C. provided BCT-100. SAB provided primary GD+ neuroblastoma cells and assisted in revising the manuscript. H.E. provided neural crest-derived cells and assisted in revising the manuscript. B.N. analysed mouse RNA-seq data.

Declaration of Interests

The authors declare no competing financial interests.

Figure Legends

Figure 1: Neuroblastoma arginine metabolism occurs in a Th1 microenvironment

A) Proliferation of tumour cell lines is inhibited by CAT1 inhibition with L-NAME, measured by ^{3}H-thymidine incorporation after 72 hours (h).

B) Cell lines were cultured with RPMI+10%FBS (R10%) or arginine-free RPMI+10%FBS (R10%-arginine). Metabolic activity was measured by MTT after 72h. n=7 replicates

C) shRNA knock-out of *ARG2* in SKNMC (high baseline ARG2 expression) and KELLY (lower baseline *ARG2* expression) decreases cell proliferation. Fold change in cell number after 72h compared to baseline. Experiment performed in duplicate. Corresponding Western blots for ARG2 in wild-type and knock-down cell lines shown below, with actin as a loading control.

D) Non-metric multi-dimension scaling of Stage I (RED) and Stage IV (BLUE) tumours shows distinct proteomic profiles for these two stages of tumours

E) Proteomic analysis of Stage I and IV tumours identifies significantly higher expression of the TH1 cytokines IL-1β, TNF-α, IFN-γ, and IL-6 in Stage IV tumours.

F) Stage I tumours express significantly higher Th2 cytokines TGF-β, IL-10, and IL-4.

G) Tissue microarrays of neuroblastoma tissue showing positive staining for IL-1β (C), TNF-α (D), IFN-γ (E) in tumour cells. Key for distribution of neuroblastoma and normal nerve tissue shown.

H) Cytokine ELISA of neuroblastoma patient plasma (n=26) at diagnosis identifies no significant differences in circulating levels of TNF-α and IFN-γ. Circulating IL-1β concentrations were significantly higher in some patients at diagnosis.

I) Expression of receptors for IL-1β (IL1R1), TNF-α (TNFR1), IFN-γ (IFNGR1), in GD2+ cells sorted from the tumours of 6 patients as examined by RT-PCR. GAPDH is shown as a loading control.

Figure 2: Tumour-infiltrating myeloid cells release IL-1β and TNF-α

A) Immunohistochemical staining of sections from neuroblastomas showing infiltration of CD33+IL-1β+ and CD33+TNF-α+ myeloid cells. Representative sections from n-27 TMA shown

B, C) Immunohistochemical staining of sections from neuroblastomas showing infiltration of CD14+ (B) and CD68+(C) myeloid cells. Representative sections shown of n=27, TMA

D) CD14+ myeloid cells from healthy donors co-cultured with sorted GD2+ tumour cells from patients upregulate CD68 expression. Representative flow cytometry shown (n=5)

E) Co-culture of CD14+ monocytes from healthy donors with tumour cell lines for 48hours leads to upregulation of IL-1β and TNF-α expression, compared to those cultured in RPMI10% media. Flow cytometry staining shown, gated on CD14+ cells. Representative staining from 3 independent experiments

F) ELISA of supernatants following co-culture of healthy donor CD14+ myeloid cells with neuroblastoma cell lines, showing increased IL-1β (upper panel) and TNF-α (lower panel) (n=5)

G) CD14+ myeloid cells from healthy donors were sorted following co-culture with neuroblastoma cell lines. Co-culture leads to increased expression of p-AKT, as shown by Western blotting (n=3)

H) Addition of AKT inhibitor MK2206 to co-cultures of CD14+ cells and neuroblastoma cell lines leads to inhibition of IL-1β (upper panel) and TNF-α (lower panel) release (n=3) protein expression.

Figure 3: Il-1β and TNF-α drive Arginase 2 expression in a p38/ERK dependent manner

A) Representative confocal microscopy of neuroblastoma cell line SKNAS shows expression of arginase 2 is increased following culture with the supernatants of neuroblastoma-conditioned monocytes. DAPI – blue, ARG2 – green, MitoTracker – red (n=3).

B) Treatment of neuroblastoma cells SKNAS and IMR32 with recombinant cytokines alone or in combination leads to upregulation of ARG2, measured by western blot. Actin is shown as a loading control. Corresponding densitometry of ARG2 relative to actin shown. Representative of n=6 replicates

C) Treatment of sorted GD2+ primary neuroblastoma cells ~~s~~(3907 and 5370) with cytokines leads to upregulation of *ARG2*, measured by Western blot. Actin is shown as a loading control. Corresponding densitometry of *ARG2* relative to actin shown.

D) Gene expression analysis shows higher expression of Arginase 2 in human neuroblastomas (n=88) compared to human neural crest-derived non-cancer primary cell lines (n=5) (http://r2.aml.nl).

E) Treatment of embryonic dorsal root ganglion stem cell line SZ16 with recombinant cytokines alone or in combination leads to upregulation of ARG2, as measured by Western blot. Actin is shown as a loading control. Corresponding densitometry of ARG2 relative to actin shown. Representative of n=3 replicates.

F) Schematic showing the signalling pathway for IL-1β and TNF-α cytokines, via ERK1/2, p38 and MSK1.

G) Time course (hours) in which IL-1β and TNF-α lead to increased p-NFκB (0.5 hours), p-ERK1/2 (from 1 hour onwards), p-p38 (0.5 hours) and p-MSK1 (0.5 hours onwards). ERK1/2, p38, and MSK1 activity are inhibited by PD90859, SB203508, and SB747651A respectively. Western blot shown. Representative of n=3 replicates.

H) Treatment of SKNAS neuroblastoma cells with recombinant cytokines leads to upregulation of ARG2, which is inhibited by ERK1/2 and p38 inhibition. Western blot shown with actin as a loading control. Corresponding densitometry of ARG2 relative to actin shown N=3 replicates

I) Treatment of SKNAS neuroblastoma cells with recombinant cytokines leads to upregulation of ARG2, which is inhibited by MSK1 inhibition. Western blot shown with actin as a loading control. Corresponding densitometry of ARG2 relative to actin shown N=3 replicates

Figure 4: Arginase 2 is mislocalised to the cytoplasm in neuroblastoma

A) Expression of Arginase 2 and mitochondrial processing peptidase (MPP) detected by western blotting in whole cell (W), mitochondrial (M) and cytoplasmic (C) fractions of cell lines. VDAC-1 is shown as a control for the mitochondrial fraction. Representative of n=5 technical replicates.

B) Arginase activity from whole cell (WCL), mitochondrial (M) and cytoplasmic (C) fractions of cell lines as measured by the conversion of arginine to urea using a colorimetric assay. Arginase in all cell fractions is active in catabolising arginine into ornithine and urea Representative of n=3 technical replicates.

C) Representative confocal microscopy of neuroblastoma cells from patients shows expression of Arginase 2 in the cell cytoplasm as well as in the mitochondria. (DAPI – blue, ARG2 – green, Mitotracker – red, yellow/orange signifies mitochondrial co-localisation, green signifies cytoplasmic localisation) Representative of n=5 repeats.

D) Immunohistochemistry shows intratumoural SAA and isotype staining for one representative human neuroblastoma. n=5 individual patients. SAA is prevalent throughout the neuroblastoma microenvironment.

E) ELISA for SAA shows significantly increased plasma SAA in all neuroblastoma patients examined (n=12), compared to healthy donors (n=12).

F) Expression of SAA receptors TLR2, TLR4, SBR2, and FPR2 on neuroblastoma cell lines as assessed by flow cytometry.

G) Treatment of SKNAS neuroblastoma cells with SAA or in combination with oxLDL did not alter ARG2 localisation as assessed by confocal. (DAPI – blue, ARG2 – green, Mitotracker – ted, yellow/orange signifies mitochondrial co-localisation, green signifies cytoplasmic localisation) Representative of n=3 repeats

H) Treatment of neuroblastoma cells with SAA and oxLDL did not alter ARG2 expression. Western blot with actin as a loading control. Representative of n=3 technical repeats with four cell lines

Figure 5: Neuroblastoma cells have a defective arginine recycling pathway

A) Schematic showing the arginine re-synthesis pathway. Arginine is transported intracellularly via Cationic Amino Acid Transporter1 (CAT1). Arginine is catabolised to ornithine and urea by arginase 2 (ARG2). Ornithine Transcarbamylase (OTC) converts ornithine to citrulline. Citrulline is converted by ArgininoSuccinate Synthetase (ASS) to argininosuccinate and back to arginine by Argininosuccinate Lyase (ASL). Deficiencies in enzyme expression lead to arginine auxotrophism.

B) RT-qPCR showing high ASS and ASL, and low OTC mRNA expression in GD2+ cells from 10 NB patients

C) Expression of arginine pathway enzymes in tumour cell lines and neural crest derived primary cells (R1113T and SZ16) by Western blotting. Actin is shown as loading control.

D) Representative immunohistochemistry staining showing neuroblastomas can be ASS positive and OTC low/negative (Patient 10) or ASS low/negative and OTC positive (Patient 11). Two representative patients of n=27 analysed.

E) Fold change in mRNA expression of ASS, OTC, CAT1 in neuroblastoma cell lines after IL-1β and TNF-α cytokine treatment (48hours), as measured by qPCR. Dotted line indicates expression in untreated cells.

F) Neuroblastoma cell lines were cultured with BCT-100 (0-4800ng/mL) for 72hours. BCT-100 leads to a decrease in neuroblastoma cell line proliferation compared to untreated cells, as measured by ^{3}H-thymdine incorporation at 72 hours. n=3.

G) Relative expression of cyclins A, B1, D1, E1 in BCT-100 treated cell lines compared to untreated controls (red line) were investigated by qPCR. n=3.

H) Neuroblastoma cell lines were cultured with BCT-100 (600ng/mL) for 72hours. The percentage of viable cells relative to untreated controls was determined by flow cytometry, using propidium iodide staining. BCT-100 leads to a decrease in neuroblastoma cell line viability. n=3.

I) PARP cleavage in whole cell lysates was determined by immunoblotting following treatment of cell lines with BCT-100 (600ng/ml) for 72 hours. Actin was used as the housekeeping gene to ensure equal loading

J) Sorted GD2+ neuroblastoma cells from patients were treated with BCT-100 (600ng/mL). Analysis of cell death was performed by transmission electron microscopy. Representative micrographs of 2 out of 6 patients shown. Upper panel – untreated cells. Lower panels –

post treatment with 600ng/mL BCT-100. Features consistent with organelle enlargement, cell membrane permeablisation, and cellular fragmentation with 600ng/mL BCT-100. Experiments performed on 3 separate occasions.

Figure 6: BCT-100 is cytotoxic to neuroblastoma and prolongs murine survival

A) Sorted GD2+ cells from TH-MYCN murine neuroblastoma s were cultured with BCT-100 (600ng/mL) for 72hours. The percentage of viable cells relative to untreated controls was determined by flow cytometry, using propidium iodide staining. BCT-100 leads to a decrease in murine neuroblastoma cell viability ex vivo

B) Immunohistochemistry staining showing murine neuroblastoma cells can express ASS or OTC. Three representative sections of n=6 tumours.

C) Plasma from control (saline) and BCT-100 treated Th-MYCN mice was collected at the start (PRE), 16 days after (MID), and at tumour end-point (END). The concentration of arginine was determined by ELISA. BCT-100 maintains a significant reduction in the plasma arginine concentration in vivo. n=6.

D,E,F) Cells were extracted from spleens (D), blood (E) and tumours (F) from tumour bearing mice and the percentage of CD3+ (T cells), Ly6G+Ly6Clow(Granulocytes), Ly6C+Ly6G-F4/80- (Monocytes), and F4/80+ cells was determined by flow cytometry .

Figure 7: Arginine depletion induces compensatory changes in tumours

A) *TH-MYCN* mice were treated with BCT-100 (60mg/kg) twice weekly intraperitoneally (ip) from the time of weaning at 3 weeks of age before overt tumour formations (Prophylaxis). Kaplan-Meier curves show that the development of tumours is significantly delayed, and that survival is increased in BCT-100 treated mice.

B) *TH-MYCN* mice were treated with BCT-100 (60mg/kg) twice weekly ip once 5 mm tumours were palpable (Treatment). Kaplan-Meier curves show a significant prolongation of survival in BCT-100 treated mice.

C) Heat-map showing the expression of arginine pathway genes from tumour bearing mice treated with BCT-100 compared to control (saline) mice. The heatmap shows z-scores of regularised log expression values

D) Tumour-bearing *TH-MYCN* mice (n=3) were treated with BCT-100 (60mg/kg iv one dose). After 7 days mice were sacrificed and tumours digested using collagenase. Immunoblotting shows a reduction in ARG2 expression following treatment. No changes in ASS1 and OTC expression are seen. Actin was used as the housekeeping gene to ensure equal loading. S= Saline control. C= Control untreated. 1,2,3 = BCT-100 treated mice.

STAR METHODS

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Arg2 antibody	Santa Cruz Biotech	Cat#sc-20151; RRID: AB_2059089
Arg2 monoclonal antibody (Clone OTI3G5)	OriGene	Cat#TA506751; RRID: AB_2623881
ASS1 antibody	Atlas Antibodies	Cat#HPA020934; RRID:AB_1845118
NOS2 (iNOS) antibody	Santa Cruz Biotech	Cat#sc-651; RRID: AB_2298577
OTC antibody	Atlas Antibodies	Cat#HPA000243; RRID: AB_1079535
CAT1 antibody	Abcam	Cat#ab37588; RRID: AB_2190720
VDAC antibody	Cell Signaling Technology	Cat#4866; RRID: AB_2272627
Phospho AKT (Ser473) antibody	Cell Signaling Technology	Cat#9271S; RRID: AB_329825
AKT antibody	Cell Signaling Technology	Cat#2938S; RRID: AB_915788
Phospho p38 MAPK (Thr180/Tyr182) antibody	Cell Signaling Technology	Cat#9215S; RRID: AB_331762
p38 MAPK antibody	Cell Signaling Technology	Cat#9212S; RRID: AB_330713
Phosho p44/p42 MAPK (ERK1/2) antibody	Cell Signaling Technology	Cat#9101S; RRID: AB_331646
p44/p42 MAPK (ERK1/2) antibody	Cell Signaling Technology	Cat#9102S; RRID: AB_330744
MPPb (PMPCB) antibody	ThermoFisher Scientific	Cat#PA5-24912; RRID: AB_2542412

OLR1 (LOX1) antibody	Sigma	Cat#AV45322; RRID: AB_1852771
PARP antibody	Cell Signaling Technology	Cat#9542; RRID: AB_2160739
LC31/LC3II	Novus Biologicals	Cat#NB6001384; RRID: AB_669581
Phospho MSK1 (Thr581) antibody	Cell Signaling Technology	Cat#9595; RRID: AB_2181783
MSK1 antibody	Cell Signaling Technology	Cat#3489; RRID: AB_2285349
Phospho NFKappa (Ser536) antibody	Cell Signaling Technology	Cat#3033S; RRID: AB_331284
NFKappa antibody	Cell Signaling Technology	Cat#8242S; RRID: AB_10859369
Actin beta	Cell Signaling Technology	Cat#4967S; RRID: AB_330288
Goat anti-Rabbit (HRP-conjugated) antibody	Cell Signaling Technology	Cat#7074S; RRID: AB_2099233
Horse anti-Mouse (HRP-conjugated) antibody	Cell Signaling Technology	Cat#7076S; RRID: AB_330924
Goat anti-Mouse (Alexa Fluor 488 conjugated) antibody	ThermoFisher Scientific	Cat#R37120; RRID: AB_2556548
Normal mouse IgG1	Santa Cruz BioTech	Cat#sc3877; RRID: AB_737222
GD2 (PE conjugated anti-Human) antibody (Clone 14G2a)	BioLegend	Cat#357302;RRID: AB_2561883
GD2 antibody	Abcam	Cat#ab68456; RRID: AB_1139816
IL1β antibody	Cell Signalling Technology	Cat#12703S; RRID:
CD68 antibody	Abcam	Cat#
CD33 antibody	Abcam	Cat#
CD14 antibody	Abcam	Cat#
TNFα antibody	Abcam	Cat#
IL1β antibody	Abcam	Cat#

INFγ antibody	Abcam	Cat#
SAA antibody	Abcam	Cat#ab655; RRID: AB_305562
TNFα (APC conjugated anti-Human) antibody	eBioscience	Cat#17734982; RRID: AB_469512
TNFα (FITC conjugated anti-Human) antibody	eBioscience	Cat#11734941; RRID: AB_10718392
SBR1/CD36L1 (PE Conjugated anti-Human) antibody	BioLegend	Cat#363203; RRID: AB_2564208
FPRL2 antibody	BioLegend	Cat#357702; RRID: AB_2561982
IL1R1 (PE Conjugated, anti-Human) antibody	R&D Systems	Cat#FAB269P; RRID: AB_2124912
IFNγR1 (PE conjugated, anti-Human) antibody	eBioscience	Cat#12119941; RRID: AB_11151326
Ly6C (FITC conjugated, anti-Mouse) antibody	BioLegend	Cat#127606; RRID: AB_1236494
Ly6G (eFlour 450 conjugated, anti-Mouse) antibody	eBioscience	Cat#48593182; RRID: AB_1548788
F4/80 (eFlour 450 conjugated, anti-Mouse) antibody	eBioscience	Cat#48480180;RRID: AB_1548756
CD3 (APC conjugated, anti-Mouse) antibody	BioLegend	Cat#100235; RRID: AB_2561455
IL1β (PE Conjugated anti-Human) antibody	eBioscience	Cat#12701881; RRID: AB_466146
CD15 (APC conjugated) antibody	eBioscience	Cat#170158; RRID: AB_2573138
CD206 (APC/Cy7 conjugated, anti-Human) antibody	BioLegend	Cat#321120; RRID: AB_2144930
CD11b (APC conjugated, anti-Human) antibody	BioLegend	Cat#982604; RRID: AB_2632619
CD68 (Alexa 488 conjugated, anti-Human) antibody	BioLegend	Cat#333812; RRID: AB_2074832

TLR2 (PE conjugated anti-Human) antibody	eBioscience	Cat#129922; RRID: AB_466262
TLR4 (PE conjugated anti-Human) antibody	eBioscience	Cat#539917; RRID: AB_1963634
TNFR1/TNFRSF1A (PE conjugated anti-human) antibody	R&D systems	Cat#FAB225P; RRID: AB_2271881
F(ab)-2 Anti-Rabbit (PE conjugated) secondary antibody	eBioscience	Cat#12473981; RRID: AB_1210761
Microbeads conjugated to anti-human CD14 antibody	Miltenyi	Cat#130050201; RRID: AB_2665482
Microbeads conjugated to anti-PE antibody	Miltenyi	Cat#130048801; RRID: AB_244373
Biological Samples		
Human: Neuroblastoma tissue and whole blood	Birmingham Children's Hospital	N/A
Human: Neuroblastoma tissue and whole blood	Children's Hospital Oxford	N/A
Human: Neuroblastoma and peripheral nerve tissue array	Biomax	Cat#NB642;
Murine: Neuroblastoma and plasma	From laboratory of Jayne Murray and David Ziegler	N/A
Murine: Neuroblastoma and plasma	From Laboratory of Louis Chesler	N/A
Chemicals, Peptides, and Recombinant Proteins		
SlowFade gold anti-fade mountant with DAPI	ThermoFisher Scientific	Cat#S36938
Type II Collagenase	Worthington Biochemicals	Cat#LS004177
LymphoPrep™	STEMCELL Technologies	Cat#07801
Human recombinant IL1β	PeproTech	Cat#200-01B
Human recombinant TNFα	PeproTech	Cat#300-01A
Human recombinant IFNγ	PeproTech	Cat#300-02
Arginine Free RPMI 1640 Medium for SILAC	ThermoFisher Scientific	Cat#88365
Arginine Free dialysed Fetal Bovine Serum (FBS)	ThermoFisher Scientific	Cat#26400036

Human Oxidised low density Lipoprotein (OxLDL)	AlfaAesar	Cat#J65591
Human recombinant Serum Amyloid A (SAA)	PeproTech	Cat#300-13
L-N^G-Nitroarginine methyl ester (L-NAME)	Cayman Chemicals	Cat#80210
PD98059 - ERK1/2 MAPK Inhibitor	Cell Signaling Technology	Cat#9900S
SB203508 - p38 MAPK inhibitor	Cell Signaling Technology	Cat#5633S
SB747651A - MSK1 inhibitor	Torcris	Cat#4630
MK-2206 - AKT Inhibitor	Cayman Chemicals	Cat#11593
Thymidine [Methyl-^{3}H]	Perkin Elmer	Cat#NET027L001MC
MegaTran transfection reagent	OriGene	Cat#TT20003
MitoTracker® Red CMXRos	ThermoFisher Scientific	Cat#M7512
cOmplete EDTA-free protease inhibitor tablets	Roche	Cat#05892791001
PhosSTOP phosphatase inhibitor tablets	Sigma	Cat#04906837001
BCT-100	Bio Cancer International	N/A
Critical Commercial Assays		
Proteomics: scioDiscover antibody microarrays	Sciomics	N/A
ELISA: Human IFNγ ELISA Max Deluxe Kit	BioLegend	Cat#430134
ELISA: Human IL1β ELISA Max Deluxe Kit	BioLegend	Cat#437004
ELISA: Human IL6 ELISA Max Deluxe Kit	BioLegend	Cat#430504
ELISA: Human IL8 ELISA Max Deluxe Kit	BioLegend	Cat#431504
ELISA: Human TNFα DuoSet ELISA Kit	R&D Systems	Cat#DY21005
ELISA: Human IL10 ELISA Max Deluxe Kit	BioLegend	Cat#430604
ELISA: Human GMCSF ELISA Max Deluxe Kit	BioLegend	Cat#432004
ELISA: Human SAA ELISA Kit	ThermoFisher Scientific	Cat#KHA0012
ELISA: IDK® Arginine ELISA Kit	Immunodiagnostik AG	Cat#K7733
Cell line validation service NSI Standard ASN-0002-2011	NorthGene	N/A
Mitochondrial Isolation Kit for Cultured Cells	ThermoFisher Scientific	Cat#89874

Lenti Concentrator Kit	OriGene	Cat#TR30025
CCK-8 – Cell counting Kit	Sigma	Cat#96992
RNAEasy RNA isolation Kit	Qiagen	Cat#74104
Reverse Transcription System	Promega	Cat#A3500
Foxp3 Fixation/Permeabilisation Concentrate and Diluent Kit	eBioscience	Cat#00552100
Experimental Models: Cell Lines		
Primary GD2+ Cells from Human Neuroblastoma patients	This Paper	N/A
Primary Human Neural Crest derived cell (SZ16)	Laboratory of Heather Etchevers (Etchevers, 2011)	N/A
Human primary, embryonic untransformed neural crest cells (R1113T)	Laboratory of Heather Etchevers (Etchevers, 2011)	N/A
SKNAS Human Neuroblastoma cell line	Laboratory of John Anderson	N/A
KELLY Human Neuroblastoma cell line	Laboratory of John Anderson	N/A
IMR32 Human Neuroblastoma cell line	Laboratory of John Anderson	N/A
LAN-1 Human Neuroblastoma cell line	Laboratory of John Anderson	N/A
SKNMC Human Ewing Sarcoma cell line	Laboratory of John Anderson	N/A
HEK293T cells		
Experimental Models: Organisms/Strains		
Mouse: Tg(Th-MYCN)41Waw	N/A	RRID:MGI:5009549
Oligonucleotides		
Primers: See Table S1 for sequences	Eurofins MWG	N/A
Probes: See Table S2 for Assay IDs and/or sequences	ThermoFisher Scientific	N/A
Recombinant DNA		

Plasmid: ARG2 Human shRNA Plasmid Kit (Locus ID 384)	OriGene	Cat#TL314692
Non-effective 29mer scrambled shRNA cassette plasmid	OriGene	Cat#TR30021
Lenti-V-pack packaging plasmid mix	OriGene	Cat#TT30037
Software and Algorithms		
ZEN 2012 Software Suite Version 8.1	Carl Zeiss Microscopy	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html
GenePix Pro 6.0 MicroArray Acquisition and Analysis software	Molecular Devices	http://mdc.custhelp.com/app/answers/detail/a_id/18691/~/genepix%C2%AE-pro-6-microarray-acquisition-%26-analysis-software-download-page
R-Bioconductor	N/A	http://www.bioconductor.org.
STRING software	N/A	https://string-db.org/
Apeiro ImageScope version 12.3.2	Leica Biosystems	https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/
FlowJo software version 10	FlowJo LLC	https://www.flowjo.com/solutions/flowjo/downloads
Linear models for microarray (LIMMA) version 3.30	Ritchie et al. (2015)	http://bioinf.wehi.edu.au/limma
ImageJ version 1.47v	National Institute of Health, USA	http://imagej.nih.gov/ij

GraphPad Prism version 6	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
ImageLab version version 6.0	BioRad	http://www.bio-rad.com/en-uk/product/image-lab-software?ID=KRE6P5E8Z
STAR RNA-Seq aligner software version 2.5.2b	Dobin et al. (2013)	https://code.google.com/archive/p/rna-star/

Contact for Reagent and Resource sharing

Further information and requests for reagents should be directed to the lead and corresponding author Dr Francis Mussai (francis.mussai@nhs.net)

Method Details

Patient Samples

Heparinised blood and tumour samples were obtained from 50 patients with neuroblastoma treated at the Birmingham Children's Hospital and Children's Hospital Oxford. GD+ neuroblastoma cells were isolated from bone marrow aspirates taken from children with stage IV disease. Samples were obtained from patients with newly diagnosed neuroblastoma, at the time of diagnostic biopsy, before the start of treatment.

Neuroblastoma murine model

Transgenic Tg(*TH-MYCN*)^{41Waw} mice were genotyped to detect the presence of human *MYCN* transgene or the Chromosome 18 insertion site, using an allelic discrimination methodology (Weiss et al., 1997, Haraguchi and Nakagawara, 2009). Specific assays were designed to measure the presence of the *MYCN* transgene (forward primer 5'-CGACCACAAGGCCCTCAGTA; reverse primer 5'-CAGCCTTGGTGTTGGAGGAG; probe 6FAM-CGCTTCTCCACAGTGACCACGTCG TAMRA) or to the site of the transgene on chromosome 18 which is disrupted during insertion (forward primer 5'-CCACAAAAATATGACTTCCTAAAAGATTT; reverse primer 5'- CATGGGACTTCCTCCTTATATGCT; probe VIC-5'-AACAATTATAACACCATTAGATATG TAMRA). After weaning, *TH-MYCN* mice were palpated for intra-abdominal tumours twice weekly. Mice with palpable tumours ranging in size between 5-20mm in diameter were then humanely sacrificed. At sacrifice, unheparinised and heparinised whole blood, as well as tumour tissue and spleen were obtained for further ex vivo analyses. Tumour tissue was processed as above. Spleens were mechanically digested

and heparinized whole blood was lysed with red blood cells lysis buffer (Qiagen). Tumour tissue, spleen and blood cell suspensions were stained with anti-mouse Ly6C, CD3, GD2 (BioLegend), Ly6G and or F4/80 (eBioscience) on ice for 30 minutes. The expression of these markers was then assessed by flow cytometry.

For treatment with BCT-100, mice were treated with 60mg/kg BCT-100 or saline, twice a week, *ip* either from weaning in the prophylaxis setting or upon the development of a 5mm tumour in the treatment setting. Mice were treated until the experimental endpoint of a 10mm abdominal tumour. In the prophylaxis experiment, mice were bled before the start of treatment, midway through the treatment, 24 hours after the fifth dose of either saline or BCT-100, and at tumour endpoint. All experimental protocols were monitored and approved by either The Institute of Cancer Research Animal Welfare and Ethical Review Body, in compliance with guidelines specified by the UK Home Office Animals (Scientific Procedures) Act 1986 and the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research or the University of New South Wales Animal Care and Ethics Committee and conducted according to the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (2013).

GD2+ tumour cell and myeloid cell isolation

For isolation of GD2+ tumour cells from human bone marrow aspirates and murine tumours, tumours were digested using Type II collagenase, labelled with anti-GD2-PE antibody and bound to anti-PE coated magnetic beads. Cells were enriched according to manufacturer's instructions (Miltenyi Biotec, Bisley, UK) to be >98% GD2+ cells as confirmed by flow cytometry using a PE conjugated anti-human GD2 antibody (BioLegend). For isolation of monocytes, peripheral blood was collected from healthy donors. Monocytes were separated using a Lymphoprep gradient (STEMCELL Technologies) and enriched by positive selection using anti-human CD14 MicroBeads (Miltenyi).

Cell lines and cultures

Human primary, embryonic untransformed neural crest (R1113T) or dorsal root ganglion derived stem cells (DRG: SZ16) were obtained and cultured as previously described (Thomas et al., 2008, de Pontual et al., 2009, Boeva et al., 2017). Neuroblastoma cell lines (SKNAS, KELLY, IMR-32, LAN-1) and the SKNMC Ewing's sarcoma cell line which has high arginase 2 expression, were routinely cultured in RPMI 1640 medium (Sigma) supplemented with 10% v/v foetal bovine serum (FBS, Sigma), 100 U/mL penicillin and streptomycin (Gibco), 1mM sodium pyruvate (Gibco) and 2mM L-Glutamine (Gibco). All cell lines were validated for authenticity by DNA short tandem repeats in line with American National Standards Institute ASN-0002-2011 (Northgene). The effects of arginine deprivation were tested on cells cultured in arginine-free RPMI 1640 for SILAC (ThermoFisher Scientific) supplemented with 10% v/v arginine-free dialysed FBS (ThermoFisher Scientific). Cells were maintained in an incubator at 5% CO_2 in air and at 37^{o}C.

Cell Treatment conditions

Where indicated recombinant human cytokines IL-1β(10 ng/ml, PeproTech), INFγ (10 ng/mL, PeproTech) and/or TNFα (10 ng/mL, PeproTech), human oxidized low density lipoprotein (50 ug/mL, OxLDL, Alfa Aesar) or of human recombinant serum amyloid A (10 µg/mL, SAA, PeproTech) were added to cell cultures for up to 48 hours. For competitive inhibition of arginine 2.5 µM L-N^G-Nitroarginine methyl ester (L-NAME, Cayman Chemicals) was added to cultures for 72 hours. ERK1/2 inhibitor PD98059 (20 µM, CST), p38 inhibitor SB203508 (20 µM CST), or MSK1 inhibitor SB747651A (10 µM, Torcris) were pre-incubated with cells for 1 hour in relevant experiments. Cellular mitochondrial metabolic activity was determined using CCK-8 (Sigma) according to manufacturer's specification.

Arginase 2 knock-down

Lentiviral plasmids encoding four unique 29-mer shRNA constructs against human arginase 2, ligated upstream to a GFP reporter in the pGFP-C-shLenti vector (#TL314692, OriGene technologies) were used for the knockdown of Arg-2 gene expression in neuroblastoma cells. An additional plasmid containing a non-effective 29-mer scrambled shRNA (#TR30021, OriGene Technologies) was used as control. Recombinant lentiviral particles carrying either the scrambled shRNA or anti Arg-2 shRNA were produced by transient co-transfection with the Lenti-V-pack packaging plasmid mix (#TR30037, OriGene Technologies) into HEK293T using the MegaTran transfection protocol (#TT20003, OriGene Technologies). Particles were harvested at 48 and 72hours post transfection, filtered through a 0.45µm cellulose acetate filter, and re-concentrated using the Lenti Concentrator kit (#TR30025, OriGene

Technologies). Cell lines were infected with re-concentrated particles at Multiplicity of Infection (MOI) of 30 for SKNMC or 40 for KELLY. GFP positive cells were selected using flow assisted cell sorting and tests performed using cell populations with >95% GFP expression. Arginase2 protein expression knockdown was confirmed by immunoblotting.

Immunoblotting

Cells were harvested, washed in PBS and lysed with RIPA lysis buffer (20nM Tris-HCl pH7.5, 150nM NaCl, 2mM EDTA, 1.0% Triton X-100) containing cOmplete™ EDTA-free protease inhibitors (Roche) and PhosSTOP™ phosphatase inhibitors (Sigma). For subcellular fractionation experiments, mitochondrial and cytosolic fractions were isolated from neuroblastoma and Acute myeloid leukaemia (AML) cells using the Mitochondrial Isolation Kit for Cultured Cells (Pierce) according to the manufacturer's protocol. Following a Bradford assay, equal amounts of total protein per treatment condition were electrophoresed on a 10% Tris-Glycine SDS-PAGE gels (BioRad) at 150V for 1 hour. Separated proteins were transferred to PVDF membranes using the TransBlot system (BioRad). Protein bands were detected using primary antibodies to ARG2 (Santa Cruz, sc-20151), iNOS (Santa Cruz, sc-651), ASS1 (Atlas antibodies, HPA020934), OTC (Atlas antibodies, HPA00243), CAT1 (Abcam, ab37588), vDAC-1 (CST, 4661T), p-AKT (CST, 9271S), AKT (CST, 2938S), p-p38 (CST, 9215S), p38 (CST, 9212S), p-ERK1/2 (CST, 9102S), ERK1/2 (CST, 9102S), MPPβ – also known as PMPCB (ThermoScientific, SA100714AE), LOX1/OLR1 (Sigma, SAB1410858), PARP and cleaved PARP (CST, 46D11), LC3I/LC3II (Novus, NB6001384), MSK (CST, 3489), P-MSK1 (CST, 9595), NFkappaB (CST, 8242S), P-NFkappaB (CST, 3033S) or beta-actin (CST, 4967S). HRP-conjugated secondary antibodies of either goat anti-rabbit (CST, 7074S) or horse anti-mouse (CST, 7076S)

were used for primary antibody detection. Blots were developed using ECL substrate (BioRad), exposed to CL-Xposure X-ray film (ThermoFisher Scientific) or documented using the ChemidocMP system (BioRad). Protein bands were quantified by densitometry analysis using ImageLab software (BioRad).

Immunofluorescence and confocal microscopy

Cells were seeded onto sterile poly-L-lysine coated 13mm diameter glass coverslip-inserts (VWR) at $1x10^4$ per coverslip placed in a 24-well plate. Seeded cells were maintained at routine culture conditions. Live staining of mitochondria was performed by adding 100nM of MitoTracker® Red CMXRos (ThermoFisher Scientific) to culture media for 15 minutes at 37^{o}C prior fixing of cells. Cells were washed in ice-cold PBS then fixed in 2% paraformaldehyde for 20 minutes at room temperature, followed by permeabilisation in 0.1% Triton-X for 10 minutes. Coverslips were incubated in blocking buffer consisting of 5% heat inactivated goat serum (HiNGS) in 1x PBS)for 1 hour. Cells were then incubated overnight with a mouse monoclonal anti-human arginase 2 antibody (Clone OTI 3G5, OriGene) diluted 1:100 in blocking buffer. The coverslips were washed twice in PBS containing 0.005% Tween-20 (PBS-T) followed by incubation with a 1:1000 dilution of Alexa Fluor-488 conjugated anti-mouse antibody (ThermoFisher scientific) for 2 hours. After three washes with PBS-T, coverslips were air dried then mounted in SlowFade gold anti-fade mountant with DAPI (ThermoFisher Scientific) to stain the cell nuclei. Cells were examined by fluorescence microscopy using a Zeiss LSM780 fluorescence confocal microscope and co-localisation analysis performed on acquired images using ZEN software suite (Carl Zeiss Microscopy).

Arginase activity assays

The activity of arginase 2 present within cells was determined by measuring the conversion of arginine into urea, as previously described (Mussai et al., 2013).

Antibody microarray analysis

Human stage I neuroblastoma tissue samples (n=13), human stage IV neuroblastoma biopsies (n=9) were analysed using scioDiscover antibody microarrays (Sciomics) which targets 900 cancer-related proteins (Schroder et al., 2013). After sample homogenisation, proteins were extracted with scioExtract buffer (Sciomics) and labelled at an adjusted concentration with scioDye 2 (Sciomics) according to the manufacturer´s instructions. A pool of all protein samples was labelled with scioDye1 and used as a reference for all experiments, allowing competitive dual-colour measurements. Array production, blocking and sample incubation were performed in compliance with strict quality control procedures as reported previously. The arrays were scanned with identical instrument laser power and adjusted PMT setting using a Powerscanner (Tecan). Spot segmentation was performed with the software GenePix Pro 6.0 (Molecular Devices).

Immunohistochemistry

Paraffin-embedded neuroblastoma sections and tissue micro-arrays (TMA) of 27 human neuroblastomas and 5 human peripheral nerve control tissue (all cores in duplicate, US Biomax) were deparaffinised and rehydrated following quality control to confirm diagnosis and antigen preservation. Tumours were stained on a Ventana Discovery Ultra automated

system, according to manufacturer's protocol. Heat-induced antigen retrieval was performed with cell conditioning 1 buffer (CC1), pH 8.5 (Ventana). Protein blocking was applied with Background Sniper (Biocare Medical, Concord, CA).

Staining with anti-human TNF-α1:150, Abcam), anti-human IL-1β (1:200, Abcam), anti-human IFN-γ (1μg/ml, Abcam) were performed at 37^{o}C, followed by the addition of secondary antibodies (Discovery anti-Rabbit HQ and anti-HQ HRP). DAB chromogen was applied (Discovery ChromoMap DAB, Ventana) and slides were counterstained with haematoxylin II (Ventana). Where indicated, detection was performed with anti-human GD2 (ab68456, Abcam), anti-human ASS1 (HPA020934, Atlas), anti-mouse ASS1 (ab170952, Abcam), anti-OTC (HPA00243, AtlasAntibodies), anti-human SAA (ab655, Abcam), anti-human CD68 (1:300, DAKO), anti-human CD33 (Abcam) aor anti-human CD14 (1:400, Abcam) using the Novolink Polymer Detection System (RE7280-K, Leica). Primary antibody incubations were carried out overnight at 4°C and tissue sections were counterstained with haematoxylin and mounted in DPX (VWR). To assess nonspecific staining, samples were similarly treated but the primary antibodies omitted and replaced with isotype specific rabbit or mouse IgG (Vector Labs Peterborough UK).

Enzyme-linked Immunosorbent Assays (ELISA)

The concentrations of cytokines IFNγ, IL-1β, TNFα, IL-6, IL-8, IL-10 and GM-CSF in plasma and cell culture media were measured by sandwich-ELISA kit according to specific manufacturer's instructions. Plasma concentrations of SAA1 protein was quantified using the human SAA ELISA Kit.

Flow cytometry

Neuroblastoma cells and myeloid cells were isolated as described above. Cell surface staining was performed with anti-human CD14, CD15, CD206, CD11b, CD68, PE anti-human TLR2, TLR4, SBR1/CD36L1, anti-human FPRL2 (BioLegend), PE anti-human IL1R1, IFNɣR1 antibodies (eBioscience) and PE anti-human TNFRSF1A/TNFR1 (LSBio). For intracellular staining, myeloid cells were stained with surface markers for 20 min at 4^{o}C followed by fixation and permeabilisation using the Foxp3 Fixation/Permeabilisation Concentrate and Diluent Kit (eBioscience). Cells were washed twice then stained with PE conjugated anti-human IL-1β and APC anti-human TNFα (eBiosciences) according to manufacturer's instructions. Cell staining was assayed on a Cyan-ADP flow cytometer (Beckman-Coulter) and analysed using FlowJo software (TreeStar Inc.). To determine cell viability after treatment cells were washed in ice-cold PBS, stained with propidium iodide (PI) and assayed on a BD Accuri C6 flow cytometer.

Monocyte Polarisation assays

Monocytes were cultured in the presence or absence of recombinant human cytokines, neuroblastoma or neuroblastoma culture supernatants (50% of final volume), with or without 0.5mM N_G-hydroxy-L-arginine (NOHA, Cayman Chemicals), and in arginine complete or arginine-free conditions for 48 hours. Where indicated 5 μM of an AKT inhibitor (MK2206, Cayman Chemicals) was added. The culture supernatants were harvested and analysed for cytokine content.

Reverse transcriptase polymerase chain reactions (RT-PCR)

Total RNA was extracted from cells using the RNeasy Kit (Qiagen) according to the manufacturers specifications (including DNase I treatment). Extracted RNA was quantified on

a NanoDrop ND-1000 spectrophotometer (ThermoScientific). First strand complimentary DNA (cDNA) was generated by incubating 1µg of extracted RNA with 500ng of random primers (Promega), 0.5mM dNTP (Promega), 1x reverse transcriptase buffer (Promega), 40U RNAse inhibitors (RNAsin, Promega) and either 100U MMLV RNAse H+ or 15U AMV reverse transcriptase (Promega). For endpoint PCR reactions, up to 100ng of sample cDNA was incubated in 5µL of 10X PCR reaction buffer (Invitrogen), 0.5mM dNTPs, one unit of *Taq* polymerase (Invitrogen), 1.5mM $MgCl_2$, 0.5µM of each forward and reverse primer and nuclease free water up to a final reaction volume of 50 µL. Human primer sequences are listed in Table S1. All quantitative real-time PCR (qPCR) were conducted on a Fast 7500 real time PCR thermal cycle (Applied Biosystems).

Transmission Electron Microscopy

Neuroblastoma cells were treated with BCT-100 (600ng/ml) in complete RPMI 1640 culture for 72hours. Cells were washed in PBS and harvested using 1X TrypleE Express Enzyme. Cells were fixed in 2.5% glutaraldehyde in PBS for 1 hour followed by secondary fixation in 1% osmium tetroxide for 1 hour. The samples were dehydrated by washing in increasing concentrations of ethanol followed by a Proylene Oxide wash. Specimens were embedded in propylene oxide/resin (1:1) mixture for 45 minutes, followed by 100% polymerisation resin at 60°C for at least 16hours. Specimens were sectioned using a diamond knife to obtain ultra-thin sections at ~80 nm in thickness then placed on 200 mesh copper slot grids for examination of cell death by transmission electron microscopy on a JEOL 1200EX electron microscope.

RNA sequencing

RNA was extracted from murine neuroblastomas described above. Samples were prepared with the Illumina TruSeq RNA Sample Preparation Kit v2 according to manufacturer's instructions and pooled libraries were sequenced on an Illumina NextSeq 500 using a 75PE sequencing strategy, over 75 cycles. Reads were mapped to the mm10 (murine) genome using STAR RNA-Seq aligner software, version 2.5.2b(12) Number of fragments per gene was counted by the same software. Differential expression analysis was performed using DESeq2 package (Love et al., 2014) Genes were ranked by the significance of the change in expression in control vs BCT-100 treated mice.

Study approval

In accordance with the Declaration of Helsinki, patient samples were obtained after written and informed consent prior to inclusion in the study. Primary human neural crest-derived stem cell lines were obtained under ethical committee approval PFS14-011 from the French Biomedical Agency for the user of embryonic material. 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.

Quantification and Statistical Analysis

Antibody microarray normalisation and statistical analysis

The acquired raw data were analysed using the linear models for microarray data (LIMMA) package of R-Bioconductor after uploading the median signal intensities. As described previously, a specialised invariant Lowess method was applied for normalisation.(Sill et al., 2010) For the differential analysis of protein expression, a one-factorial linear model was fitted with LIMMA resulting in a two-sided t-test or F-test based on moderated statistics. Differences in protein abundance between sample groups are presented as log-fold changes (logFC) calculated for the basis 2. The presented p-values were adjusted for multiple testing by controlling the false discovery rate according to Benjamini and Hochberg. In all comparisons, proteins were defined as significantly differential with a log-fold change above 0.5 or below −0.5 and an adjusted p-value below 0.05. Functional enrichment analyses were conducted with the STRING software (https://string-db.org) for the proteins with significantly differential abundance between groups, whereby up- and downregulated proteins were analysed separately.

Arginase 2 Fluorescence Intensity

Quantification of cell-by-cell fluorescence intensity for Arginase 2 expression across treatment conditions were performed using ImageJ software (National Institute of Health, USA). Briefly confocal image stacks were converted to single channel images. Pixel intensity measurements were determined form single channel Images representing Arginase 2 staining with Image thresholds set to match positive structures within defined cell boundaries.

Statistical analysis

Parametric student t-Tests were used to determine the statistical significance of the difference in paired observations between groups (GraphPad Prism, USA). All p values are two-tailed and p values <0.05 were considered to represent statistically significant events. Significance was recorded as * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Document S1 – Supplementary Figures S1 – S7

TABLE S1: Oligonucleotide primers and probes used in this study

Oligonucleotide & Probes	Source
MYCN transgene primer, Forward: CGACCACAAGGCCCTCAGTA	Hadjidaniel and Reynolds (2010)
MYCN transgene primer, Reverse: - CAGCCTTGGTGTTGGAGGAG	Hadjidaniel and Reynolds (2010)
MYCN transgene probe: 6FAM™- CGCTTCTCCACAGTGACCACGTCG-TAMRA™	Hadjidaniel and Reynolds (2010)
Murine: Chromosome 18 insertion site Forward: CCACAAAAATATGACTTCCTAAAAGATTT	This Paper
Murine: Chromosome 18 insertion site Reverse: CATGGGACTTCCTCCTTATATGCT	This Paper
Murine: Chromosome 18 insertion site probe: VIC™-5'- AACAATTATAACACCATTAGATATG-TAMRA™	This Paper
Human: Cyclin A primer, Forward: AATGGGCAGTACAGGAGGAC	Mussai et al. (2015b)
Human: Cyclin A primer, Reverse: CCACAGTCAGGGAGTGCTTT	Mussai et al. (2015b)
Human: Cyclin B1 primer, Forward: CATGGTGCACTTTCCTCCTT	Mussai et al. (2015b)

Human: Cyclin B1 primer, Reverse: AGGTAATGTTGTAGAGTTGGTGTCC	Mussai et al. (2015b)
Human: Cyclin E primer, Forward: GGCCAAAATCGACAGGAC	Mussai et al. (2015b)
Human: Cyclin E primer, Reverse: GGGTCTGCACAGACTGCAT	Mussai et al. (2015b)
Human: Cyclin D primer, Forward: GAAGATCGTCGCCACCTG	Mussai et al. (2015b)
Human: Cyclin D primer, Reverse: GACCTCCTCCTCGCACTTCT	Mussai et al. (2015b)
Human: Cyclin B2 primer, Forward: GCGTTGGCATTATGGATCG	Mussai et al. (2015b)
Human: Cyclin B2 primer, Reverse: TCTTCCGGGAAACTGGCTG	Mussai et al. (2015b)
Human: Arginase 2 primer, Forward: ATGTCCCTAAGGGGCAGCCTCTCGCGT	Mussai et al. (2015b)
Human: Arginase 2 primer, Reverse: ATGTCCCTAAGGGGCAGCCTCTCGCGT	Mussai et al. (2015b)
Human: NOS2 (iNOS) primer, Forward: CCTCAAGCTATCGAATTTGTC	Mussai et al. (2015b)
Human: NOS2 (iNOS) primer, Reverse: TTGCCATTGTTGGTGGAGTA	Mussai et al. (2015b)
Human: ASS1 primer, Forward: GGGGTCCCTGTGAAGGTGACC	Mussai et al. (2015b)
Human: ASS1 primer, Reverse: CGTTCATGCTCACCAGCTC	Mussai et al. (2015b)
Human: OTC primer, Forward: AGTTATTTAACCAGCGTGTTT	Mussai et al. (2015b)
Human: OTC primer, Reverse: TCCCCCTAAAGTGAATAAGTG	Mussai et al. (2015b)
Human: CAT1 primer, Forward: ATGGGTGGAAACGCTGATGATAC	Mussai et al. (2015b)
Human: CAT1 primer, Reverse: ACCTTGCCTGTTAAGTCTGGGTG	Mussai et al. (2015b)

Human: CAT2A primer, Forward: TTAACACTTATGATGCCGTACTACCT	Mussai et al. (2015b)
Human: CAT2A primer, Reverse: GCAACTGGTGACTGCCTCTTACT	Mussai et al. (2015b)
Human: CAT2B primer, Forward: ATGCCTCGTGTAATCTATGCTATG	Mussai et al. (2015b)
Human: CAT2B primer, Reverse: ACTGCACCCGATGATAAAGTAGC	Mussai et al. (2015b)
Human: GAPDH primer, Forward: CCAGCCGAGCCACATCGCTC	Mussai et al. (2015b)
Human: GAPDH primer, Reverse: ATGAGCCCCAGCCTTCTC	Mussai et al. (2015b)

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