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Targeting glutamine metabolism enhances tumor specific immunity by modulating suppressive myeloid cells

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Conflict of interest

J.D.P., B.S.S, R.R. and P.M. are scientific founders of Dracen Pharmaceuticals and possess equity. Technology arising in part from the studies described herein were patented by Johns Hopkins University and subsequently licensed to Dracen Pharmaceuticals.
Summary

Myeloid cells comprise a major component of the tumor-microenvironment (TME) promoting tumor growth and immune evasion. By employing a small molecule inhibitor of glutamine metabolism, not only were we able to inhibit tumor growth, but we markedly inhibited the generation and recruitment of myeloid-derived suppressor cells (MDSCs). Targeting tumor glutamine metabolism led to a decrease in CSF3 and hence recruitment of MDSCs as well immunogenic cell death leading to an increase in inflammatory tumor-associated macrophages (TAMs). Alternatively, inhibiting glutamine metabolism of the MDSCs themselves led to activation induced cell death and conversion of MDSCs to inflammatory macrophages. Surprisingly, blocking glutamine metabolism also inhibited IDO expression of both the tumor and myeloid derived cells leading to a marked decrease in kynurenine levels. This in turn inhibited the development of metastasis and further enhanced anti-tumor immunity. Indeed, targeting glutamine metabolism rendered checkpoint blockade-resistant tumors susceptible to immunotherapy. Overall, our studies define an intimate interplay between the unique metabolism of tumors and the metabolism of suppressive immune cells.

Keywords

glutamine, tumor associated macrophages, IDO, immunometabolism, MDSC, immunotherapy
Introduction

The prodigious growth of tumor cells demands specialized metabolic reprogramming. Tumor metabolism not only promotes growth but also creates a TME that inhibits immune effector function by depleting critical metabolites (such as tryptophan, glucose and glutamine) and generating inhibitory metabolites (such as kynurenine). Alternatively, suppressive immune cells, that are metabolically distinct from effector cells, thrive in the TME (1-3). To this end, the most prominent immune cells in the TME are suppressive macrophages.

Macrophages, which constitute a major component of tumors, are involved in cancer initiation, progression, angiogenesis, metastasis, and creating an immune suppressive environment (4-7). Additionally, TAMs express metabolic enzymes like iNOS or arginase 1 (both enzymes that lead to arginine depletion) and IDO (an enzyme that leads to tryptophan depletion) that inhibits T cell activation and proliferation (8-11). TAMs also express PDL1 and PDL2, which interact with PD1 on T cells. These interactions trigger inhibitory immune checkpoint signals on the T cells (12).

In addition to TAMs, MDSCs also play important roles in creating an immunosuppressive TME (13). In mice, MDSCs express Gr1 (Ly6C and Ly6G) and CD11b. These markers define two subsets of MDSCs, polymorphonuclear-MDSCs (PMN-MDSCs, CD11b+ Ly6Clo Ly6G+) and monocytic-MDSCs (Mo-MDSCs, CD11b+ Ly6Chi Ly6Gneg). Though there are no distinct markers to distinguish between MDSCs and the tumor associated neutrophils (TANs)/monocytes at different stages of maturity, they are both functionally immunosuppressive cells in the TME (14-16). Akin to TAMs, MDSCs also express enzymes that deplete key nutrients from T cells such as iNOS, arginase 1, PDL1/2, SLC7A11 and secrete suppressive cytokines (17-19). Importantly, these cells do not highly express MHC and co-stimulatory molecules, which are essential for antigen presentation and activation to cytotoxic T cells (20).

Glutamine metabolism as a whole is a crucial element of cancer cell metabolism. Glutamine is important for nucleotide synthesis, amino acid production, redox balance, glycosylation, extracellular matrix production, autophagy, and epigenetics (3, 21). In this report, we employed a small molecule – a prodrug of 6-Diazo-5-oxo-L-norleucine (DON) (22) to target glutamine metabolism. Our studies reveal that
blocking glutamine metabolism markedly inhibits the generation and recruitment of MDSCs and promotes the generation of anti-tumor inflammatory TAMs. Mechanistically, we demonstrate a tumor specific and myeloid cell specific role for glutamine in promoting the immunosuppressive TME.
Results

*Targeting glutamine metabolism inhibits tumor growth by altering suppressive myeloid cells in an immunotherapy-resistant model of triple negative breast cancer.*

The 4T1 triple negative breast cancer model is resistant to checkpoint blockade and this lack of response is associated with a low frequency of mutations and an abundant presence of suppressive myeloid cells such as MDSCs, TAMs, and TANs (23). In agreement with previous reports, 4T1 tumors were resistant to treatment with anti-PD1, anti-CTLA4, or the combination of anti-PD1 and anti-CTLA4 (Figure 1A and 1B).

Glutamine is a critical anaplerotic substrate for anabolic growth that is necessary for the specialized Warburg metabolism that facilitates robust tumor growth (3, 21). Therefore, we employed a glutamine metabolism inhibitor prodrug of 6-Diazo-5-oxo-L-norleucine (DON), referred to as JHU083 (Figure 1C) (22). 4T1 tumor-bearing mice were treated with JHU083 (1 mg/kg) for 7 days starting at day 7 after tumor inoculation followed by a lower dose (0.3 mg/kg) until the mice were sacrificed. We observed a marked decrease in the growth of the 4T1 tumors following treatment with the glutamine antagonist, JHU083, as compared to the vehicle (referred to as a NT group), anti-PD1, anti-CTLA4, or the combination of anti-PD1 and anti-CTLA4 (Figure 1A and 1B) treated groups. At this dosing regimen, treatment with the DON prodrug was well tolerated as we did not observe any weight loss due to treatment with JHU083 (Supplementary Figure 1A).

In the 4T1 model, untreated mice begin to appear ill at day 30 due to metastasis to the lungs. Thus, we wondered whether treatment with the glutamine antagonist, in addition to inhibiting the growth of the primary tumor might also be inhibiting metastasis. Strikingly, at this time point there was marked inhibition of lung metastasis in the treated mice (Figure 1D-F). The ability of JHU083 to inhibit metastasis was also observed when we delivered tumor via tail vein injection (Supplementary Figure 1B).

To dissect the potential contribution of immune cells in inhibiting tumor growth and metastasis in the treated mice, we analyzed the efficacy of JHU083 in tumor-bearing recombination activating gene 1 (RAG1) knock out (KO) mice (lacking adaptive immunity with intact innate immunity) or non-obese...
diabetic severely combined immune-deficient interleukin-2 receptor gamma-chain null (NSG) mice (lacking adaptive immunity as well as defective innate immunity). JHU083 was able to inhibit tumor growth equally in the RAG1 KO mice and wild type (WT) mice upon initial treatment (Figure 1G). However, with time, JHU083 treated RAG1 KO mice showed faster tumor growth compared to the treated WT mice. These data suggest that while the adaptive endogenous response to 4T1 is minimal (hence the lack of efficacy of checkpoint blockade) there is in fact some form of adaptive immunity in this model (Figure 1G). On the other hand, JHU083 treated NSG mice showed minimal therapeutic benefit when compared to JHU083 treated RAG1 KO and WT mice in the early and late phase of tumor growth (Figure 1G). While RAG1 mice eliminate the contribution of T and B cells, they still possess innate (macrophage and NK cell mediated) anti-tumor mechanisms as compared to NSG mice. To investigate the contribution of NK cells in this model, we depleted NK cells using an anti-asialo-GM1 depletion antibody in RAG1 KO mice. Although the depleting antibody rapidly decreased NK cells, we did not see any differences in tumor growth between JHU083 treated RAG1 KO mice versus JHU083 treated RAG1 KO mice with NK cell depletion (Supplementary Figure 1C). Given the fact that NSG mice have defective macrophages and that macrophages are a major component of the TME compared to RAG1 KO mice, these data suggested to us that blocking glutamine metabolism might be inhibiting tumor growth (and metastasis) in part by regulating the myeloid immune response (Figure 1G and Supplementary Figure 1C).

**Targeting glutamine metabolism inhibits the infiltration of MDSCs in both primary tumor and metastatic sites.**

We wanted to test the hypothesis that targeting glutamine metabolism might not only directly arrest tumor growth but also mitigate the generation, recruitment or reprogramming of suppressive myeloid cells. In light of the robust generation of MDSCs in the 4T1 model, we examined the effect of immune checkpoint blockade or glutamine antagonism on MDSCs. As expected, 4T1 tumor-bearing mice showed elevated MDSCs in the blood compared to tumor free mice (Figure 2A). Treatment with immune checkpoint
blockade had no impact on the recruitment of PMN-MDSCs and Mo-MDSCs in both the peripheral blood and the tumor (Figure 2A and B).

On the other hand, the percentages of both PMN-MDSCs and Mo-MDSCs were markedly reduced amongst the tumor-infiltrating immune cells of JHU083 treated tumor-bearing mice compared to the control group in both the peripheral blood and the tumor (Figure 2A and B). Concomitant with the reduction of the percentage of MDSCs within the tumors, we also observed a decrease in the absolute numbers of these cells in the treated versus the untreated mice (Figure 2C). Additionally, we tested the ability of glutamine antagonism to inhibit MDSCs in another immunotherapy resistant tumor model, the Lewis lung carcinoma (3LL) model. Similar to the 4T1 model, targeting glutamine metabolism in 3LL tumor-bearing mice led to improved control of tumor growth as well as a decrease in the percentage of MDSCs (Supplementary Figure 2A and B). Overall these findings demonstrate that targeting glutamine metabolism not only inhibits tumor growth but also leads to a decrease in MDSCs. However, importantly, the absolute number of TAMs per tumor weight did not change (Figure 2D). That is, treatment with JHU083 did not lead to a decrease in all myeloid cells within the TME, but rather led to the selective depletion of MDSCs.

MDSCs are believed to play a crucial role in facilitating metastasis (24, 25). Since we observed reduced metastasis in the lung with glutamine antagonism, we interrogated infiltration of MDSCs into the lungs from the JHU083 treated and untreated mice. Specifically, we interrogated the lungs on day 14, at a time point when there is no macroscopic evidence of metastasis in either the untreated or the treated mice. Similar to the primary tumors, we observed a marked decrease in the absolute numbers of MDSCs in the whole lung of JHU083 treated mice compared to untreated mice (Figure 2E). Therefore, the inhibition of glutamine metabolism blocks the generation and recruitment of MDSCs within the primary TMEs as well as at sites of metastasis, thus markedly inhibiting the development of metastatic foci.

_Targeting glutamine metabolism inhibits MDSCs by increasing cell death and decreasing tumor CSF3 expression._
To understand the effects of glutamine metabolism on the decrease in MDSCs, we evaluated the direct effect of glutamine antagonism on MDSCs. When we treated MDSCs with DON (the active form of JHU083) in vitro, we observed an increase in apoptosis as defined by active caspase-3 (Figure 3A). Next, we evaluated the induction of MDSC apoptosis in vivo. To minimize the possibility that differences in tumor size itself affects MDSC numbers, we treated mice with JHU083 for a short duration after the tumors were fully established (17 days after tumor inoculation). Consistent with our in vitro finding, we also observed significantly increased active caspase-3 on both PMN-MDSCs and Mo-MDSCs within 33 hours after JHU083 treatment in the blood (Figure 3B). Thus, these data suggest that glutamine antagonism directly affects apoptosis of MDSCs in the blood. Surprisingly, however, within 7 hours of treatment we observed markedly decreased tumor infiltrating MDSCs (Figure 3C). This finding led us to hypothesize in addition to inducing apoptosis in MDSCs, blocking glutamine metabolism might affect the recruitment of MDSCs.

In this regard, several studies have demonstrated that increased secretion of growth factors such as CSF1 (M-CSF), CSF2 (GM-CSF), and CSF3 (G-CSF) promote the recruitment of MDSCs to the TME (26). As such, we wanted to determine if targeting glutamine metabolism inhibited MDSCs in the TME in part by limiting the elaboration of these critical growth factors. Indeed, we observed markedly decreased CSF1, CSF2, and CSF3 levels in DON treated 4T1 tumor culture supernatants (Supplementary Figure 3A-C). To confirm this finding with an in vivo model, we measured CSF1, CSF2, and CSF3 in the serum of 4T1 tumor-bearing mice treated with or without JHU083. Compared to the vehicle group, the glutamine antagonist treated mice demonstrated reduced CSF3 in circulating serum and within tumor lysate (Figure 3D). However, CSF1 and CSF2 were undetectable in circulating serum (data not shown). To test the role of CSF3 in the recruitment of MDSCs in this model, we implanted CSF3 overexpressing (OE) 4T1 tumors (Supplementary Figure 3C). Consistent with previous reports (27-29), mice implanted with CSF3 OE 4T1 tumors recruit more PMN-MDSCs compared to the empty vector (EV) 4T1 tumor implanted mice (Figure 3E). This indicates that CSF3 is a critical factor for the recruitment of PMN-MDSCs in 4T1 tumors. Additionally, glutamine antagonism reduces MDSCs in both EV and CSF3 OE 4T1 implanted mice (Figure
Furthermore, in vitro DON treated 4T1 cells and in vivo whole tumor lysate showed reduced CsF3 mRNA expression (Figure 3D). Such findings support the notion that one mechanism by which glutamine antagonism leads to a decrease in MDSCs within the TME is through inhibiting the transcriptional expression of CSF3 from tumor cells.

To dissect the mechanism of transcriptional regulation of CSF3 in tumor cells, we evaluated the expression of C/EBPβ (LAP), a well described transcription factor that promotes CSF3 expression (27-29). Indeed, the expression of C/EBPβ was reduced in JHU083 treated 4T1 tumor lysates and in DON treated in vitro 4T1 cells (Figure 3F). To confirm the role of C/EBPβ in the recruitment of PMN-MDSCs in this model, we implanted mice with C/EBPβ Overexpressing (OE) 4T1 tumors (Supplementary Figure 3D). Similar to the CSF3 OE tumor-bearing mice, C/EBPβ OE tumors recruited more PMN-MDSCs compared to EV tumor-bearing mice (Figure 3G). Also, glutamine antagonism inhibits MDSCs recruitment in both EV and C/EBPβ OE 4T1 tumor-bearing mice (Figure 3G). Thus, these data suggest that glutamine metabolism is important for the maintenance of C/EBPβ, which is crucial for CSF3 expression that promotes PMN-MDSCs recruitment.

Overall, our data thus far demonstrate that targeting glutamine metabolism, in addition to inhibiting tumor growth, has a robust effect on inhibiting MDSCs in immunotherapy resistant tumors. Mechanistically this is through two distinct effects: i: Direct effects on MDSC promoting caspase-3 dependent cell death. ii: Effects on the tumor with reduction of CSF3 by inhibiting C/EBPβ transcription factor activity.

**Targeting glutamine metabolism promotes the reprogramming of tumor associated macrophages.**

While targeting glutamine metabolism inhibited the recruitment of Mo-MDSCs and PMN-MDSCs to the tumor, it did not “wholesale” inhibit myeloid cells. Recall, we did not observe significant differences in the amount of TAMs within the tumors from JHU083 treated and untreated mice (Figure 2D and Figure 4A). Thus, we were interested in understanding the effect of JHU083 on the phenotype and function of the TAMs. To this end, we performed RNA sequencing on sorted TAMs from vehicle and JHU083 treated 4T1 tumor-bearing mice. More than 3,000 significant mRNA transcripts were differentially expressed (Figure
As expected, we found significant downregulated genes between TAMs from vehicle and JHU083 treated mice within glutamine related pathways, such as DNA replication, cell cycle, pyrimidine metabolism, pentose phosphate pathway, glycolysis, purine metabolism, and arginine and proline metabolism (Table 1).

Notably, by evaluating the GO pathway analysis for biological processes, we found that lysosome and toll-like receptor (TLR) signaling related genes are significantly upregulated with drug treatment between TAMs isolated from tumors of JHU083 treated and untreated mice (Table 2). Furthermore, using gene set enrichment analysis (GSEA), we found an upregulation of phagocytic vesicles and pattern recognition receptor signaling activity related genes in TAMs from the treated mice (Figure 4C). Specifically, the expression of genes encoding for TNF, TLR4, CD80, and CD86 molecules related to a pro-inflammatory phenotype were increased while Il10 and Nos2 gene expression, which are known to inhibit anti-tumor T cell responses, were decreased (Figure 4D).

To confirm our RNA sequencing data, we performed flow cytometry to phenotype the TAMs within the TME in 4T1 tumor-bearing mice. We observed increased surface TLR4, MHCII, CD80, CD86 and reduced iNOS on TAMs from the JHU083 treated mice (Figure 4E). Overall, our findings demonstrate that targeting glutamine metabolism promotes a pro-inflammatory phenotype amongst TAMs.

Multiple recent studies have demonstrated that pro-inflammatory TAMs inhibit tumor growth (30, 31). Our RNA sequencing data of TAMs from JHU083 treated mice demonstrated an increase in the pro-inflammatory cytokine, Tnf. In agreement with our RNA seq data, we also observed increased TNF protein production in TAMs from JHU083 treated mice (Figure 4F). After in vitro LPS stimulation for 9 hours, further enhancement of TNF production was also observed in TAMs from JHU083 treated mice compared to TAMs from vehicle treated mice (Figure 4F). Furthermore, there was a negative linear correlation between TNF production and tumor weight, thereby suggesting pro-inflammatory macrophages regulates tumor progression (Figure 4G). Additionally, depletion of TNF abrogated in part the JHU083 mediated anti-tumor effects (Figure 4G).
A previous report demonstrated that glutamine deprivation further induces M1 polarization mediated by increased NF-κB signaling (32). To confirm this finding, we treated bone marrow-derived macrophages (BMDMs) with varying doses of DON during LPS stimulation. After 24 hours, we observed increased TNF secretion in DON treated BMDMs along with increased NF-κB nuclear localization (Supplementary Fig 4A). On the other hand, we observed decreased IL-10 secretion and phosphorylation of STAT3 (Supplementary Fig 4B). Thus, this finding suggests that glutamine inhibition enhances a pro-inflammatory macrophage phenotype that might be due to increased NF-κB and reduced STAT3 signaling.

**Targeting glutamine metabolism promotes the conversion of MDSCs to pro-inflammatory tumor associated macrophages.**

Given the observation that glutamine antagonism promotes the reprogramming of TAMs (Figure 4C-F), we hypothesized that the recruited MDSCs in the tumor might also be converted into pro-inflammatory macrophages. To this end, isolated MDSCs from the blood of CD45.1 4T1 tumor-bearing mice (21 days after 4T1 tumor inoculation) were adoptively transferred into CD45.2 4T1 tumor-bearing mice (7 days after 4T1 tumor inoculation). The MDSC recipient CD45.2 4T1 tumor-bearing mice were treated with JHU083 for 7 days, then tumors were harvested (Figure 5A). As seen in Figure 4F, we observed increased TNF secretion from endogenous TAMs (CD45.2\(^+\) cells) in JHU083 treated mice with LPS stimulation (Figure 5B). More strikingly, we found significantly increased TNF production from the adoptively transferred CD45.1\(^+\) cells in tumor from JHU083 treated CD45.2 4T1 tumor-bearing mice (Figure 5B). Additionally, we observed increased activation markers such as MHCII, CD80, and CD86 on the transferred CD45.1\(^+\) cells in the JHU083 treated mice (Figure 5C). These observations support a model whereby glutamine antagonism enhances pro-inflammatory TAM differentiation not only just in TAMs but also in converted TAMs from MDSCs as shown by the adoptive transfer experiments.

**The glutamine antagonist JHU083 enhances immunogenic tumor cell death and antigen presentation of TAMs.**
Though we observed intrinsic enhancement of pro-inflammatory macrophage phenotypes with glutamine inhibition upon LPS stimulation, it was unclear how TAMs were activated in the TME with glutamine antagonist treatment without LPS stimulation (Figure 4E-F and 5B-C). Previous reports have shown that immunogenic tumor cell death (ICD) induces TLR signaling in TAMs through the release of Danger-Associated Molecular Patterns (DAMPs). Increased endoplasmic reticulum stress and reactive oxygen species (ROS) production are important mediators in inducing ICD (34). Thus, we investigated the ability of JHU083 to promote a pro-inflammatory TME by inducing ICD. Indeed, treatment of 4T1 cells with DON led to an increase in ROS and active caspase-3 in vitro (Figure 6A and 6B). In addition, targeting glutamine metabolism of 4T1 tumor cells both in vitro and in vivo led to an increase in surface exposure of calreticulin, an endoplasmic reticulum protein and a DAMP (Figure 6C). To explore this concept further, we cultured BMDM cells in conditioned media from DON-treated tumor cell supernatants. Increased p-NF-κB (ser536) (TLR downstream signaling) and LAMP2 (lysosome marker) were observed in BMDMs cultured in DON-treated tumor conditioned media compared to vehicle treated tumor conditioned media (Figure 6D). This result suggests that tumor cell death induces macrophage activation mediated by downstream TLR signaling and lysosome function, which correlated with the RNA sequencing data (Table 2).

Next, we tested whether the increased NF-κB signaling and lysosome function by ICD indeed increased antigen presentation to T cells. To test this idea, BMDMs were co-cultured with the B16 OVA melanoma tumor with various doses of DON for 24 hours. After removing and washing away the media, cell proliferation dye-labeled naïve CD8\(^+\) T cells from OTI mice were co-cultured with the BMDMs and tumor cells (Figure 6E). Next, the OVA-specific cytotoxic T cell populations were analyzed by flow cytometry. We observed that the co-culture cells with DON showed increased OVA-specific T cell proliferation when compared to the vehicle-treated group (Figure 6F). This finding also held true using a MC38 OVA tumor model (Supplementary Figure 5A). When we added DON to the macrophages prior to the co-culture, we did not observe proliferating CD8\(^+\) T cells despite increased MHCII expression with glutamine inhibitor treatment (Supplementary Figure 5B). This finding demonstrates that macrophages
require danger signals to trigger proper antigen presentation to T cells. On the other hand, when we added DON to the tumor cells prior to the co-culture, we observed increased proliferating CD8\(^+\) T cells (Supplementary Figure 5B). However, it was less effective compared to the co-culture of macrophages and tumor cells both with DON treatment. Thus, glutamine inhibition exerts a synergistic effect between immunogenic tumor cell death and enhanced activation of macrophages by glutamine inhibition itself (Supplementary Figure 5B). Furthermore, when we co-cultured T cells with BMDMs from MyD88/TRIF (downstream of TLR signaling) or TFEB (key regulator of lysosomal biogenesis) deficient mice, there was diminished T cell proliferation (Figure 6G). Thus, DON-induced tumor cell death enhances antigen presentation (as determined by T cell proliferation) in a MyD88/TRIF signaling and lysosome dependent fashion.

**The glutamine antagonist JHU083 enhances cross-presentation by TAMs.**

To dissect whether this enhanced cross-presentation to CD8\(^+\) T cells by BMDMs with JHU083 treatment is mediated by macrophages or by some differentiated inflammatory dendritic cells (DCs) from BMDMs, we performed the same in vitro experiment on Batf3 KO BMDMs which have cross-presenting deficient DCs. Like WT BMDMs, Batf3 KO BMDMs equally showed enhanced cross-presentation with DON treatment (Figure 7A). This data suggests that macrophages can cross-present tumor antigens to CD8\(^+\) T cells when they are co-cultured with glutamine antagonist treated tumor cells.

In an in vivo setting, as expected, MC38 tumors grew faster in Batf3 KO mice compared to WT mice (Figure 7B). Nevertheless, JHU083 treatment still led to a reduction in tumor size in Batf3 KO mice, indicating cross-presentation can still occur independent of fully functional cross-presenting DCs (Figure 7B). Additionally, in agreement with our recent report (35), the percentage of tumor infiltrating CD8\(^+\) T cells from JHU083 treated WT mice were increased within the tumor, and these cells demonstrated enhanced activation and cytotoxic markers (CD44 and granzyme B) and the proliferation marker, Ki67 (Figure 7C). Although there was a significant decrease in the CD8\(^+\) T cells in Batf3 KO mice, JHU083 treatment could still increase the percentage and enhance the phenotype of CD8\(^+\) T cells in the KO mice.
That is, even in the absence of cross-presenting DCs, JHU083 promoted the CD8\(^+\) T cell anti-tumor response.

**The glutamine antagonist JHU083 enhances the efficacy of immune-checkpoint blockade in immunotherapy resistant tumor.**

Our studies demonstrate that targeting glutamine metabolism boosts immune responses by reprogramming tumor metabolism, enhancing a pro-inflammatory phenotype of TAMs, reducing MDSCs, and promoting ICD. Furthermore, we observed an increase in the tumor-infiltrating CD8\(^+\) T cells, and an enhanced ratio of CD8\(^+\) T cells to MDSCs ratio from JHU083 treated 4T1 tumor-bearing mice compared to control group (Figure 8A). More importantly, we also observed an increase in the CD8:MDSCs ratios and percentages of CD8\(^+\) T cells in the lungs of the treated versus untreated mice, suggesting enhanced anti-tumor immunity by glutamine metabolism inhibition in both primary tumor and metastatic sites (Figure 8A). Thus, we were interested in determining if blocking tumor glutamine metabolism could enhance the efficacy of checkpoint blockade. First, we tested this hypothesis on a checkpoint blockade sensitive tumor. We assessed the ability of JHU083 to inhibit the growth of EO771, which is similar to 4T1 triple negative breast cancer and is moderately sensitive to immunotherapy in the form of anti-PD1 monotherapy (31). We observed significant inhibition of tumor growth and enhanced survival with JHU083 treatment alone (Supplementary Figure 6A and B). Notably, the combination of JHU083 + anti-PD1 or JHU083 + anti-PD1 + anti-CTLA4 resulted in greater inhibition of tumor growth and enhanced survival, suggesting an additive effect of combining metabolic therapy with checkpoint blockade (Supplementary Figure 6A and 6B). Thus, in this sensitive tumor model of checkpoint blockade, glutamine antagonism serves to enhance the efficacy of immunotherapy.

Next, we evaluated whether JHU083 can enhance the efficacy of checkpoint blockade in the 4T1 tumor model. Recall, 4T1 tumor cells are extremely resistant to immunotherapy in the form of checkpoint blockade (Figure 1A and 1B). To this end, mice were injected with 4T1 tumors and treated on day 7 post injection with either vehicle, JHU083 alone, anti-PD1+ anti-CTLA4, or JHU083 + anti-PD1 + anti-CTLA4. The mice treated with anti-CTLA4 + anti-PD1 had no therapeutic benefit compared to the vehicle treated
group as seen in Figure 1A and 1B (Figure 8B and C). The JHU083-treated group displayed delayed tumor growth and an increase in survival (Figure 8B and C). When mice were treated with JHU083, anti-PD1 and anti-CTLA4, we observed further attenuation of tumor growth and an increase in overall survival compared to the JHU083 monotherapy group (Figure 8B and 8C).

Taken together, these data demonstrate that glutamine inhibition induces ICD of tumor cells by increasing ROS, which leads to an increase in MyD88/TRIF-dependent signaling, lysosomal function, and antigen presentation in TAMs, and subsequently enhanced tumor-specific effector T cell proliferation. Furthermore, by altering the TME, glutamine inhibition can enhance the efficacy of checkpoint blockade even in tumors that are resistant to immunotherapy (Figure 8D).

**Targeting glutamine metabolism reshapes the metabolism of the tumor.**

Our data demonstrates the direct effects of targeting glutamine metabolism on tumor and suppressive myeloid cells. However, inhibition of glutamine metabolism can also affect the TME itself that can alter the infiltration and function of immune cells. Thus, we hypothesized that blocking glutamine metabolism would not only inhibit tumor growth but also alter the metabolic milieu of the TME. To this end, we performed targeted metabolomics using liquid-chromatography tandem mass spectrometry (LC-MS) on equal weighted 4T1 tumors from vehicle and JHU083-treated mice to assess the effects of glutamine inhibition on cell metabolism. Metabolomics analysis with LC-MS revealed two distinct metabolic clusters, which correlated to the two experimental groups (Figure 9A and 9B). As expected, glutamine inhibition caused reduced TCA cycle intermediates and less conversion of glutamine to glutamate, resulting in an increased glutamine/glutamate ratio, implying glutaminase (GLS) inhibition (Supplementary Figure 7A). In addition, we also observed significant changes in citrulline, N-carbamoyl-L-aspartate, thymine, S-adenosyl-L-methionine, homoserine, guanosine, nicotinamide ribotide, hydroxyproline, succinate, cystathionine, aspartate, uridine, Acetyl-L-lysine, and Dimethyl-L-arginine (Figure 9B, 9C and Supplementary Figure 7B). Using pathway enrichment analysis, we found significant differences between the metabolites in tumors from vehicle and JHU083 treated mice, such as in glycine and serine metabolism,
phosphatidylcholine biosynthesis, methionine metabolism, urea cycle, glutamate metabolism, ammonia recycling, amino sugar metabolism, and arginine and proline metabolism pathways (Supplementary Figure 7C).

**Targeting glutamine metabolism alters the metabolism of the tumor and the site of metastasis in both glutamine dependent and glutamine independent pathways.**

Since targeting glutamine metabolism reduced MDSCs recruitment in the lungs before visible metastasis occurs (Figure 2E), we wanted to know whether the metabolic milieu of the TME at the lung metastatic site also plays a critical role in facilitating metastasis. To this end, we also performed metabolomics on the lungs from the JHU083 treated and untreated mice on day 17, before visible metastasis occurs, to understand the possible metabolic changes related to the development of metastasis. Similar to the primary tumors, LC-MS analysis of the lungs revealed two distinct metabolic clusters (Figure 10A and 10B). That is, despite a lack of macroscopic metastasis in the lungs on day 17, we observed significant metabolic changes (Figure 10B and Supplementary Figure 8). These data suggest that blocking glutamine metabolism would not only alter the metabolic milieu of the TME but also change the metabolism of the metastatic site.

To distinguish the unique and shared metabolites within the primary tumor and metastatic sites, we analyzed significantly altered metabolites from the primary tumor and lungs. We found several shared metabolites such as thymine, hydroxyproline, citrulline, thiamine, citraconoic acid, allantoin and kynurenine (Figure 10C).

Interestingly, of the 200 molecules tested, kynurenine was found to be the most reduced metabolite in the treated mice in the primary tumor (Figure 9C and 10D). This was quite surprising since glutamine is not known to be directly involved in kynurenine metabolism. Strikingly, in agreement with our primary tumor data, the kynurenine level was also markedly reduced in the lungs from the JHU083-treated mice (Figure 9B and 10D). These data indicate that blocking glutamine metabolism can inhibit lung metastasis by altering the metabolism of both the primary tumor and the site of metastasis.
Targeting glutamine metabolism reduces IDO expression by regulating STAT1 and STAT3 signaling.

The enzyme IDO metabolizes tryptophan to kynurenine which is a potent inhibitor of T cell proliferation, and function and its expression is actively regulated by signals within the TME (36). Thus, we hypothesized that glutamine antagonism regulates IDO expression but not its enzymatic activity. Indeed, IDO protein expression in 4T1 tumor lysates was decreased in the JHU083 treated mice compared to vehicle treated mice (Figure 11A). Similarly, we observed markedly reduced IDO expression in JHU083 treated MC38 tumor lysates (Figure 11B). To determine the major source of IDO in the tumor, we performed immunoblotting on different cell populations in the TME. We observed that tumor cells are a major source of IDO and JHU083 treatment dramatically decreases tumor IDO expression (Figure 11C). Additionally, sorted MDSCs and TAMs express IDO that is also markedly reduced with JHU083 treatment (Figure 11D). Furthermore, the ratio of kynurenine: tryptophan was markedly diminished in the tumors from the JHU083 treated mice (Figure 11E).

IDO is transcriptionally regulated by STAT1 and STAT3. To understand how IDO expression is regulated by glutamine metabolism, we measured the phosphorylation of STAT1 as an indicator of its transcriptional activity. With DON treatment, we observed reduced p-STAT1 in the tumor along with reduced IDO expression upon IFNγ stimulation (Figure 11F). Accordingly, reduced mRNA expression of IDO was observed with glutamine inhibition (Figure 11F). Similarly, with DON treatment, we observed reduced p-STAT3 in the RAW264.7 macrophage cell line along with decreased IDO protein and mRNA expression upon IFNγ stimulation (Figure 11G). Thus, targeting glutamine metabolism not only inhibits glutamine dependent pathways, but also leads to a marked decrease in p-STAT1 and p-STAT3 dependent IDO expression resulting in a robust reversal of the kynurenine: tryptophan ratio.

Interestingly, the metabolite kynurenine has been implicated in promoting metastasis (37-39). Indeed, we observed higher IDO expression in the lungs from untreated mice with tumors prior to the appearance of metastases compared to the lungs of tumor-free mice. Similar to the primary tumor, JHU083 treatment decreased IDO expression in the lung (Figure 11H). Overall, these data suggest that the robust
ability of targeting glutamine metabolism to inhibit metastasis may be attributed in part to altering the “metabolic” and immunologic metastatic microenvironment.

Finally, to explore the potential link between glutamine metabolism and IDO expression in patients with breast cancer, we analyzed The Cancer Genome Atlas (TCGA) using normal and breast invasive carcinoma data. TCGA analysis revealed significant correlation between expression levels of IDO and glutamine utilizing enzymes which are inhibited by DON, such as cytidine triphosphate synthase (CTS1), guanine monophosphate synthase (GMPS), GLS1, asparagine synthetase (ASNS) and CAD (carbamoyl-phosphate synthetase). These correlations are only found in breast carcinoma samples but not normal tissue controls, indicating the potential link between glutamine metabolism and IDO expression levels in breast cancer (Figure 11I).

Together, our findings elucidate fundamental insight into the role of glutamine metabolism in not only regulating tumor growth and the TME but also controlling the generation and recruitment of MDSCs, reprogramming of TAMs and regulating IDO expression, which result in inhibition of cancer growth, metastasis, and immune evasion. Further, we demonstrate the ability of our small molecule inhibitor of glutamine metabolism to render immunotherapy resistant tumors susceptible to checkpoint blockade (Figure 12).
Discussion

It is becoming increasingly clear that specialized tumor metabolism is not merely to support the growth and energetics of cancer cells, but also plays a critical role in creating an immunosuppressive TME. To this end, the metabolic program of suppressive myeloid cells is specialized in order to thrive within the TME. We demonstrated that targeting glutamine metabolism not only inhibits tumor growth but also alters the TME and subsequently tumor immune evasion. Therefore, targeting glutamine metabolism led to: i. inhibition of tumor derived CSF3, ii. inhibition of tumor IDO expression resulting in decreased kynurenine, iii. immunogenic cell death of tumor cells, iv. apoptosis of MDSCs v. conversion of MDSCs to inflammatory macrophages, vi. enhanced activation of macrophages and antigen presentation. The net results of these effects were decreased tumor growth/metastasis and enhanced anti-tumor immunity. Importantly, our studies reveal the intimate relationship between the metabolism of tumor cells and the metabolism of suppressive immune cells and how targeting glutamine metabolism can enhance tumor infiltration by immune cells.

The critical role of glutamine in supporting the prodigious anabolic requirements of cancer cells has been appreciated for some time (3, 40, 41). The current efforts of targeting glutamine in tumors has primarily focused on the initial step of glutaminolysis through the development of selective GLS inhibitors (42-44). While such inhibitors demonstrate robust efficacy in vitro, it is becoming clear that GLS inhibition is far less effective in vivo (45-48). As such, we have developed a pro-drug of the glutamine antagonist DON, which not only inhibits glutaminase, but also all other glutamine requiring reactions important to tumor growth including purine and pyrimidine biosynthesis, redox control, glycosylation, amino acid and collagen synthesis, autophagy and epigenetic modification (3, 21). DON as an anti-tumor agent has been studied for 60 years. While DON resulted in some encouraging responses in phase I and II clinical trials in the 1950s to the 1980s, the development of DON was limited by its GI toxicity (49, 50). Our compound, JHU083, limits toxicity by creating an inert prodrug that is preferentially (though not exclusively) converted to the active compound DON within the TME (22, 50). It is important to note, that while we can evaluate the efficacy of our approach in mice, we cannot evaluate the toxicity and pharmacokinetics in small animals.
(rodents), because they metabolize the prodrugs differently than humans (22). As such, our dosing schedule in mice is much more limited by the potential toxicities than it would be in humans. Nonetheless, even though JHU083 is rapidly converted to DON in mice, we have identified a robust therapeutic window to evaluate its effects on tumor growth and the TME. Furthermore, unlike the previous clinical trials employing DON, in the modern era, our studies provide robust clinical rationale for developing combination regimens using our DON prodrug along with immunotherapy.

While the specialized metabolism of tumors promotes growth it also profoundly influences the TME. Indeed, the hypoxic, acidic, nutrient depleted TME in and of itself serves to inhibit anti-tumor immune responses. Such an environment favors the residency of suppressive myeloid cells such as MDSCs, TANs, and TAMs, all of which contribute to promoting tumor growth, angiogenesis, metastasis, and immune escape (51). Additionally, suppressive myeloid cells contribute to resistance against immune checkpoint blockade (52, 53). Our data demonstrate that targeting glutamine metabolism leads to a marked decrease in MDSCs in both the peripheral blood of tumor-bearing mice and within the tumors itself. Mechanistically this is due in part to i. increased caspase-3 dependent cell death, ii. decreased secretion of CSF3 from the tumors by reducing the expression of transcription factor C/EBPβ, and iii. promoted MDSCs differentiation into pro-inflammatory TAMs. Interestingly, glutamine antagonism did not simply reduce the percentage and absolute numbers of TAMs within the tumor. Rather, it promoted the generation of pro-inflammatory TAMs. Analogous with our findings, a recent study demonstrated that glutamine depletion enhances M1 and reduces M2 macrophage phenotype and function (32).

A recent study demonstrated that the inhibition of aerobic glycolysis in tumors can reduce MDSCs recruitment through the reduction of CSF3 secretion via enhanced C/EBPβ degradation (29). The authors showed that degradation of C/EBPβ is mediated by enhanced autophagy. Similarly, we recently demonstrated that glutamine antagonism inhibits aerobic glycolysis in tumors (35). Also, glutamine deprivation or inhibition enhances autophagy and reduces mTOR activity in tumors (54, 55). As such our preliminary studies suggest that the ability of JHU083 to down modulate C/EBPβ protein and thus the expression of CSF3 may be due in part to the ability of JHU083 to promote autophagy.
In as much as glutamine plays a critical role in multiple anabolic metabolic pathways, we hypothesized that glutamine antagonism would drastically alter the “metabolic” TME. Indeed, in 4T1 tumors from the JHU083 treated mice, we observed decreased metabolites: citrulline, N-carbamoyl-L-aspartate, thymine, S-adenosyl-L-methionine, homoserine, guanosine, nicotinamide ribotide, hydroxyproline and succinate. Surprisingly, of the 200 metabolites queried, kynurenine was the most differentially regulated. Kynurenine is the product of tryptophan metabolism by IDO and has potent immunosuppressive effects. IDO knockout mice robustly reject tumors and inhibitors of IDO are being developed clinically as immunotherapy (36, 56, 57). Unexpectedly, JHU083 inhibited conversion of tryptophan to kynurenine. However, its mechanism of action was not by directly inhibiting IDO but rather by inducing the down modulation of IDO gene expression via reduced STAT1 and STAT3 transcriptional activity.

In addition to inhibiting growth of the primary tumor, glutamine antagonism proved to be potent in inhibiting the development of metastasis. This observation has important clinical relevance to many tumors (especially breast cancer) where metastatic spread of the primary tumor negates successful surgical removal. In the 4T1 model, a major site of metastasis is the lung. Interestingly, we observed both metabolic and immunologic differences in the lungs of untreated and treated mice even in the absence of macroscopic metastasis. MDSCs are thought to play an integral role in promoting metastasis (26, 58, 59). It has been shown that MDSCs increase angiogenesis, tumor invasion, and formation of a pre-metastatic niche by enhancing pro-angiogenetic factors (such as VEGF, PDGF, b-FGF, and angiopoietins), MMPs, and chemokines (such as CXCL1, CXCL2, MCP1 and CXCL5) (26). To this end, we observed an increase in the CD8+ T cells: MDSCs ratio in the lungs of the treated mice even in the absence of observable tumor. Likewise, kynurenine levels were decreased in the lungs of JHU083 treated mice compared to untreated mice even before there was evidence of macroscopic metastasis. Previous studies have shown that kynurenine can promote metastasis by inducing epithelial-to-mesenchymal transition by activating the aryl hydrocarbon receptor (39).
Immunotherapy in the form of anti-PD1 and anti-CTLA4 has revolutionized our approach to treat certain cancers. Yet, in spite of these successes it is clear that not all cancers respond to checkpoint blockade alone and even amongst responsive cancers, not all patients respond (52, 60-62). Such observations point to multiple different mechanisms of tumor immune evasion. Our data suggest that by targeting glutamine metabolism we can enhance the efficacy of immunotherapy. To this end, in the anti-PD1 responsive EO771 model, the addition of JHU083 enhanced the overall response of checkpoint blockade. Furthermore, in the 4T1 model that was resistant to combined anti-PD1 and anti-CTLA4 treatment, we could overcome resistance in part by blocking glutamine metabolism. Overall, these observations support the view that tumor metabolism represents a means by which cancer cells can evade anti-tumor immune responses. Further, we provide the preclinical rationale for strategies targeting glutamine metabolism as a means of enhancing immunotherapy for cancer.
Methods

Further details are provided in the supplemental material.

Animal

C57BL/6J, CD45.1 BALB/cJ, OTI, RAG1 KO, Batf3 KO and BALB/cJ (both male and female mice, 6-8 weeks of age) were purchased from Jackson Laboratories. Mice were randomly assigned to experimental groups, and ages and genders were matched. NSG mice were obtained from the Johns Hopkins Animal resources facility. MyD88/TRIF double KO mice were kindly provided by Dr. Franck Housseau (Johns Hopkins University, Baltimore) (63, 64). TFEB KO mice were kindly provided by Dr. Andrea Ballabio (Baylor College of Medicine, Houston) (65).

GEO: The RNA sequencing data have been deposited in the GEO under ID codes GSE119733.

Statistics

Graphs were generated and statistical analysis were performed with Prism 7 (GraphPad). Comparison between two means was done by 2-tailed t-test or non-parametric 2-tailed Mann-Whitney t-test. Comparisons between three or more means were done by 1-way ANOVA test with multiple comparisons post-test. Survival test was done by Log-rank (Mantel-Cox) test. The association between two ranked variables was done by spearman rank correlation. A P value less than 0.05 was considered significant.

Study approval

The Institutional Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD) approved all animal protocols.
Author Contributions


Acknowledgements

We thank the members of the Horton and Powell labs for review of this manuscript. And we thank Dr. T.C. Wu for his kind gift of mice. This work was supported by NIH grants (R01CA229451 to J.D.P. and B.S.S. and S10 OD016374 to the JHU Microscopy Facility), R01HL141490 to M.R.H. and grants R01AI077610, R01CA226765 to J.D.P.) the Bloomberg–Kimmel Institute for Cancer Immunotherapy to J.D.P. and B.S.S., Under Armour Women’s Health & Breast Cancer Innovation Grants to J.D.P.


Table 1. Gene ontology analysis of RNA sequencing data on sorted TAMs from WT and JHU083 treated 4T1 tumor-bearing mice. Molecular functional analysis using gene ontology (GO) in downregulated genes (q value < 0.05)

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Table 2. Gene ontology analysis of RNA sequencing data on sorted TAMs from WT and JHU083 treated 4T1 tumor-bearing mice. Molecular functional analysis using gene ontology (GO) in upregulated genes (q value < 0.05)

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Figure 1

(A) Tumor volume (mm³) over Days Post 4T1 injection for NT, aPD1, aCTLA4, aPD1+aCTLA4, and JHU083.

(B) Tumor weight (mg) comparison with NT, aPD1, aCTLA4, aPD1+aCTLA4, and JHU083.

(C) JHU083 chemical structure.

(D) Spontaneous 4T1 lung metastasis showing NT 4T1 D30 Lung and JHU083 4T1 D30 Lung.

(E) Nodule numbers for NT and JHU083.

(F) Spontaneous 4T1 lung metastasis images: NT 4T1 D30 Lung and JHU083 4T1 D30 Lung.

(G) Tumor volume (mm³) over Days Post 4T1 injection for WT NT, WT JHU083, RAG1 KO NT, RAG1 KO JHU083, NSG NT, and NSG JHU083.

% Survival over Days Post 4T1 injection for WT NT, WT JHU083, RAG1 KO NT, RAG1 KO JHU083, NSG NT, and NSG JHU083.
Figure 1. Glutamine antagonism inhibits tumor growth and lung metastasis in an immune dependent manner. (A-F) 1x10^5 4T1 cells were implanted subcutaneously into the mammary fat pad of BALB/cJ female mice. On day 7, 10, 13, 17, and 24, mice were injected IP with 250 μg anti-PD1 and/or 100 μg anti-CTLA4 antibodies. 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. (A) Tumor size was monitored (N=5/group). (B) On day 17, tumor weight was measured. (C) The structure of the glutamine antagonist prodrug, JHU083. 6-Diazo-5-oxo-L-norleucine (DON) is depicted in black and its ethyl and 2-Amino-4-methylpentanamido promoieties are depicted in blue and red, respectively. (D-F) The whole lungs were harvested, and spontaneous lung metastasis were analyzed. (D-E) To quantify tumor nodules, on day 30, lungs were inflated with 15% india ink. (D) Representative picture of lungs. (E) Quantification of tumor nodules in lungs (N=16-19/group, three experiments combined). (F) Representative histology sections stained with H&E. (G) 1x10^5 4T1 cells were implanted subcutaneously into the mammary fat pad in WT BALB/cJ, RAG1 KO, or NSG female mice. 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) daily starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. Tumor burden and survival were assessed (N=5/group). Data are representative of at least three independent experiments. NS: no significant. **p<0.001, ****P<0.001, Mean ± S.D. Two way ANOVA with Tukey multiple comparisons post-test (A), Kruskal-Wallis test with Dunn's multiple comparisons post-test (B), Mann-Whitney tests (E) and Log-rank (Mantel-Cox) test (G).
Figure 2. Glutamine antagonism inhibits infiltration of MDSCs in both primary tumor and lung metastatic site. 1x10^5 4T1 cells were implanted subcutaneously into the mammary fat pad of BALB/cJ female mice. On day 7, 10, 13, 17, and 24, mice were injected IP with 250 μg anti-PD1 and/or 100 μg anti-CTLA4 antibodies. 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used (N=5/group). (A) On day 17, percentages of PMN-MDSCs (CD11b^+F4/80^negLy6C^loLy6G^hi) and Mo-MDSCs (CD11b^+F4/80^negLy6C^hiLy6G^neg) of live cells from the blood were analyzed by flow cytometry (N=5/group). (B) On day 14, tumors were harvested and tumor-infiltrating immune cells were analyzed by flow cytometry. The populations of PMN-MDSCs and Mo-MDSCs were shown. (C) Each cell population numbers were counted. Total cell numbers were divided by respective tumor weights (mg). (N=5-10/group) were shown. (D) The number of TAMs (CD11b^+F4/80^CD8^negLy6C^negLy6G^neg) per mg was shown. (E) On day 14, lungs from subcutaneously injected 4T1 tumor-bearing mice were harvested. PMN-MDSCs and Mo-MDSCs among lung-infiltrating immune cells were counted. (N=3/group) Data are representative of at least three independent experiments. NS: no significant. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, Mean ± S.D. One way ANOVA with Tukey multiple comparisons post-test (A, B and E). Mann-Whitney tests (C and D).
Figure 3

(A) MDSCs

![Active caspase-3](image)

Active caspase-3

Actin

(B) 4T1 Blood 33hrs

PMN-MDSCs

4T1 Blood 33hrs

Mo-MDSCs

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Active caspase-3 % normalized to NT

(C) TIL MDSCs 7hrs

Cell numbers (X105)

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% PMN-MDSCs of live CD45+

(D) In vivo 4T1 Serum

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Serum CSF3 (O.D. Value)

(E) CSF3 OE

4T1 PMN-MDSCs

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% PMN-MDSCs of live CD45+

(F) In vivo Sorted GFP+ 4T1

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C/EBPβ OE

4T1 PMN-MDSCs

In vitro 24hrs

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% PMN-MDSCs of live CD45+
Figure 3. Glutamine antagonism reduces MDSCs by increasing cell death and inhibiting tumor CSF3 secretion. (A) MDSCs from 4T1 tumor-bearing mice were treated with DON (1µM) for 24hrs, and active caspase-3 level was analyzed by immunoblot. Actin was used as loading control. (B-D) 4T1 tumor-bearing mice were treated with JHU083 (1 mg/kg) starting at day 14 after tumor inoculation. (B) After 7hrs of first treatment and following every day treatment, active caspase-3 on PMN-MDSCs and Mo-MDSCs from blood were analyzed by flow cytometry at the indicated time point (N=5/group). (C) Cell numbers and percentages of MDSCs from tumor were counted and analyzed by flow cytometry (N=5/group). (D) Serum (N=16/group) and tumor (N=4/group) were collected from 4T1 tumor-bearing mice and CSF3 was measured by ELISA (top). After 6hrs treatment with or without DON (1µM), Csf3 mRNA levels were measured in 4T1 cells (bottom left) (N=3, technical replication). Csf3 mRNA levels were measured from in vivo 4T1 tumor lysates by q-PCR (N=5 mice) (bottom right). (E) Percentages of PMN-MDSCs and Mo-MDSCs from empty vector (EV) 4T1 or CSF3 overexpressed (OE) 4T1 tumor bearing mice were analyzed by flow cytometry. (F) C/EBPβ levels were measured on GFP+ sorted tumor cells from 4T1 tumor bearing mice (left) and on 4T1 tumor cells with or without DON (1µM) treated for 24hrs (right) by immunoblotting. (G) PMN-MDSCs and Mo-MDSCs from EV 4T1 or C/EBPβ OE 4T1 tumor bearing mice were analyzed by flow cytometry. Data are representative of at least two (E-G) or three (A-D) independent experiments. NS: no significant. *P<0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, Mean ± S.D. Two-way ANOVA with post multiple t tests (B, C, E and G). Unpaired t tests (D).
Figure 4. Glutamine antagonism induces reprogramming of TAMs from a suppressive to a pro-inflammatory phenotype. (A) The percentages of TAMs from vehicle or JHU083 treated 4T1 tumor-bearing mice (on day 17). (B) Volcano plot showing significant changes in gene expression (red) from RNA sequencing analysis on NT and JHU083 treated TAMs (CD11b+F4/80+7AADnegLy6CnegLy6GnegCD8neg) from 4T1 tumor-bearing mice (on day 14). q-value <0.05. (C) Gene set enrichment analysis (GSEA) plot of phagocytic vesicle and pattern recognition receptor signaling activity related genes in NT vs. JHU083 on TAMs. Enrichment scores in gene set is shown. (D) Normalized gene expression from RNA sequencing analysis on NT (black) and JHU083 (red) treated TAMs from 4T1 tumor-bearing mice (on day 17). All genes are significant (q-value <0.05). (E) Representative histogram and summary graphs of TLR4, MHCII, CD86, CD80 and iNOS expression on TAMs. (F) TILs were harvested on day 17 from 4T1 tumor-bearing mice treated with or without JHU083. Cells were incubated with golgi-plug in the presence or absence of LPS for 9 hours ex-vivo. Percentages of TNF+ cells were analyzed by flow cytometry (left). MFI of TNF from TNF+ cells (right). (G) Correlation of the % TNF+ secreting of TAMs after stimulation with respective to tumor weight (left). 4T1 tumor-bearing mice were treated with JHU083 every day and intraperitoneally injected with isotype antibody or 100µg a-TNF antibody (depleting) twice per week starting at day 7 after tumor inoculation (right). Data are from one experiment with 5 mice per group (A-D) or from three independent experiment with 5-10 mice per group (E-G). NS: no significant. *P < 0.05, ****P<0.001, Mean ± S.D. Mann-Whitney tests (A). Unpaired t test (E). One way ANOVA with Tukey multiple comparisons post-test (F) and spearman correlation (G).
Figure 5

(A) Blood MDSCs (90%) – from CD45.1 mice

CD45.1 mice 4T1 tumor inoculation 4T1 tumor inoculation Adoptive transfer MDSCs from CD45.1 tumor bearing mice CD45.2 recipient mice

-21days -7days 0days 7days

JHU083 (1mg/kg/day x D0-D6)

(B) CD45.2+ Endogenous TAMs

(C) CD45.2+ MDSC -->TAMs conversion

+LPS (ex vivo)

+LPS (ex vivo)

MHCII on CD45.1

CD86 on CD45.1

CD80 on CD45.1

NTJHU083
Figure 5. Glutamine antagonism induces differentiation of MDSCs from a suppressive to a pro-inflammatory phenotype. Isolated MDSCs in blood from CD45.1 4T1 tumor bearing mice (21 days after 4T1 tumor inoculation) were adoptively transferred into CD45.2 4T1 tumor bearing mice (7 days after 4T1 tumor inoculation). Then, MDSC recipient CD45.2 4T1 tumor bearing mice were treated with JHU083 until harvesting tumors on day 7. (A) Schematic of the experiment. (B) Cells were incubated with golgi-plug in the presence or absence of LPS for 9 hours ex vivo. Percentages of TNF^+ CD45.2^+ cells (endogenous, left) and TNF^+ CD45.1^+ cells (adoptively transferred, right) were analyzed by flow cytometry. (C) Summary graph of MHCII, CD86 and CD80 gMFI. Data are from three independent experiment with 5-10 mice per group (B, C). *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, Mean ± S.D. One way ANOVA with Tukey multiple comparisons post-test (B). Unpaired t test (C).
Figure 6

(A) Graph showing CellRox gMFI with DON (μM) concentrations of 0, 0.5, 5, and 50.

(B) Western blot analysis showing active caspase-3 and actin levels.

(C) Graph showing percent surface calreticulin of total cell with NT, DON 1μM, DON 5μM, and DON 10μM concentrations.

(D) Graph showing surface calreticulin gMFI on GFP+ 4T1 tumor cells with DON concentrations of 1, 5, and 10 μM.

(E) +/− DON (1 or 5 μM) for 18hrs

(F) Graph showing % Divided cells of CD8+ with NT, DON 1μM, and DON 5μM concentrations.

(G) Proliferation dye for WT BMDM, MyD88/TRIF DKO BMDM, and TFEB KO BMDM with DON concentrations of 1, 5 μM.
Figure 6. Glutamine antagonism increases immunogenic cell death and antigen presentation to T cells. (A-C) 4T1 tumor cells were cultured with or without DON (0, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM) for 24 hours. (A) Cells were harvested and stained with CellROX (ROS measurement), and analyzed by flow cytometry. Representative histogram (left) and summary graph (right). (B) Cells were lysed and active caspase-3 was analyzed by immunoblot. (C) Cells were stained for calreticulin and analyzed by flow cytometry. Percentages of surface calreticulin+ were shown (left). Summary graph of surface calreticulin gMFI on GFP+ gated in vivo tumor cells (right). (D) 3LL cells were cultured with or without DON (0.5 μM or 1 μM). After 1 hour of incubation, cells were washed and replaced with drug-free media. After 24 hours, supernatants were harvested and used as conditioned media (CM). BMDMs were cultured in the presence of these conditioned media for 24 hours. Phospho-NF-κB (ser536) and LAMP2 were measured by immunoblot. (E-G) 3x10^5 BMDMs and 5x10^4 B16-OVA tumor cells were co-cultured with or without DON (1 μM or 5 μM). After 24 hours of incubation, supernatants were discarded and 3x10^5 eFluor450-labeled naïve CD8+ T cells from OTI mice were added. (E) Schematic of the experiment. (F) Representative flow plot (left) and percentages of divided cells (right) from CD8+ T cells were analyzed by flow cytometry. (G) BMDMs from WT, MyD88/TRIF double KO (DKO) or TFEB KO mice and B16-OVA tumor cells were co-cultured in the same method as (E), and histogram of the dilution of eFluor450-labeled CD8+ T cells is shown. Data are representative of at least three independent experiments. *P < 0.05, ** P < 0.01, **** P < 0.001, Mean ± S.D. One way ANOVA with Tukey multiple comparisons post-test (A and F). Mann-Whitney tests (C).
**Figure 7**

(A) Proliferation dye

(B) % of change in tumor volume after treatment

(C) % CD8+TCRβ+/CD45+ vs CD44, Granzyme B, Ki67
Figure 7. Glutamine antagonism increases tumor antigen cross-presentation to T cells by macrophage. (A) 3x10^5 WT or Batf3 KO BMDMs and 5x10^4 B16-OVA tumor cells were co-cultured in the presence or absence of 1 μM of DON. After 24 hours of incubation, supernatants were discarded and 3x10^5 eFluor450-labeled naïve CD8+ T cells from OTI mice were added. Histogram of divided cells from CD8+ T cells were analyzed by flow cytometry. (B-C) 5x10^5 MC38 cells were implanted subcutaneously into flank of WT C57BL/6J mice or Batf3 KO mice. MC38 tumor-bearing mice were treated with JHU083 (0.3 mg/kg) daily starting at day 14 after tumor inoculation. (B) Tumor weight was recorded (left), and percent change in tumor volume was calculated (right). (C) On day 21, tumors were harvested, and CD8+ T cells were analyzed by flow cytometry. Data are representative of three independent experiments. *P < 0.05, **P < 0.01, ***p < 0.005, ****P < 0.001, Mean ± S.D. One way ANOVA with Tukey multiple comparisons post-test (B). Unpaired t tests (C).
Figure 8

(A) 4T1 TIL

(B) 4T1 TIL

(C) Lung

(D) Lung

(E) 4T1 TIL

(F) 4T1 TIL

(G) 4T1 TIL

(H) 4T1 TIL

(I) 4T1 TIL

(J) 4T1 TIL

(K) 4T1 TIL

(L) 4T1 TIL

(M) 4T1 TIL

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OO 4T1 TIL

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QQ 4T1 TIL

RR 4T1 TIL

SS 4T1 TIL

TT 4T1 TIL

UU 4T1 TIL

VV 4T1 TIL

WW 4T1 TIL

XX 4T1 TIL

YY 4T1 TIL

ZZ 4T1 TIL
Figure 8. Glutamine antagonism enhances the efficacy of immune-checkpoint blockade in immunotherapy resistant tumor. (A) Percentages of CD8$^+$ cells and ratio of CD8$^+$ T cells to MDSCs from tumor and lung from subcutaneously injected 4T1 tumor bearing mice were analyzed by flow cytometry (N=5-10/group). (B-C) 4T1 tumor-bearing mice were treated with vehicle or JHU083 alone, or vehicle or JHU083 in combination with 250 μg anti-PD1 and 100 μg of anti-CTLA4 antibodies (On day 7, 10, 13, 17, and 24). (B) Tumor sizes and (C) survival curves were recorded. (D) Proposed model. Data are representative of three independent experiments. *P < 0.05, **P < 0.01, ****P < 0.001, Mean ± S.D. Mann-Whitney t tests (A). Log-rank (Mantel-Cox) test (C).
Figure 9

(A) Tumor Scores plot

(B) Tumor

(C) JHU083 vs NT Tumor

JHU083 vs NT

Log2 (FC) -Log10 (P)

NT JHU083

guanosine
methylmalonic acid
succinate
allantoin
hydroxyproline
kynurenine
homoserine
neopterin ribotide
thymine
UDP-D-glucose
UDP-N-acetyl-glucosamine
ethanolamine
pyrocatecholic acid
glutamine
serine
acetylcarnitine
pyruvate
aspartate
glycine
N-acetyl-glutamate
N6-acetyl-L-lysine
uridine
flavone
Ng,NG-dimethyl-L-arginine
D-glucosamine-6-phosphate

JHU083 (19.4%)

PC2 (43.1%)

PC1
Figure 9. Glutamine antagonism alters metabolism of primary tumor and metastatic sites in glutamine dependent and independent pathways. 1x10^5 4T1 cells were implanted subcutaneously into mammary fat pad of BALB/cJ female mice. 4T1 tumor-bearing mice were treated with JHU083 (1 mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. On day 17, tumors were harvested, and equal mass of tumor samples were subjected for LC-MS analysis (A) Principal component (PC) analysis between NT (vehicle treated, green) and JHU083-treated (pink) groups. (B) Heatmap visualization of the metabolite changes between NT (green) and JHU083-treated (red) groups. (C) Volcano plot of metabolites were shown. Log2 fold change vs. –log10 (FDR corrected P value) representing significant metabolite changes. Pink: significant. Data are from one experiment with 4-5 mice per group. t test (B, C).
Figure 10

(A) Lung Scores plot

(B) Lung

(C) Tumor

(D) Tumor Kynurenine

Lung Kynurenine

-2 0 2

NT JHU083

N-acetylputrescine
thymine
citracnic acid
thiamine
kynurenine
4-aminobutyrate
UDP-D-glucose
glycerate
ADP
adenosine
N-acetyl-glucosamine-1-phosphate
cystathionine
thiamine
D-glucosamine-1-phosphate
creatine
glycerophosphocholine
homoserine
S-methyl-5-thioadenosine
xanthine
nicotinamide
carboxymethyl phosphate
inosine
methylthionine
anthraniolate
myo-inositol

homoserine
N-carbamoyl-L-aspartate
3-phospho-serine
guanosine
7-methylguanosine
nicotinamide ribotilde
thiamine-phosphate
riboflavin
S-adenosyl-L-methionine
succinate
methylimelanic acid
anthranilate
allantoate
xanthosine
2-hydroxyglutarate

kynurenine
thymine
citracnic acid
citulline

taurine
guanosine
7-methylguanosine
nicotinamide ribotide
thiamine-phosphate
riboflavin
S-adenosyl-L-methionine
succinate
methylimelanic acid
anthranilate
allantoate
xanthosine
2-hydroxyglutarate

kynurenine
thymine
citracnic acid
citulline

taurine
guanosine
7-methylguanosine
nicotinamide ribotide
thiamine-phosphate
riboflavin
S-adenosyl-L-methionine
succinate
methylimelanic acid
anthranilate
allantoate
xanthosine
2-hydroxyglutarate

kynurenine
thymine
citracnic acid
citulline

taurine
guanosine
7-methylguanosine
nicotinamide ribotide
thiamine-phosphate
riboflavin
S-adenosyl-L-methionine
succinate
methylimelanic acid
anthranilate
allantoate
xanthosine
2-hydroxyglutarate
Figure 10. Glutamine antagonism alters metabolism of metastatic sites in glutamine dependent and independent pathways. 1x10⁵ 4T1 cells were implanted subcutaneously into mammary fat pad of BALB/cJ female mice. 4T1 tumor-bearing mice were treated with JHU083 (1 mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. On day 17, whole lungs from mice were harvested, and whole lung lysates were subjected for LC-MS analysis. (A) Principal component analysis between the vehicle treated (NT) (green) and JHU083 (pink) treated lungs and (B) heatmap visualization of the metabolite changes between NT (green) and JHU083 (red) treated group were shown. (C) Venn diagram displaying shared significantly changed (1.5 fold, p<0.05) metabolites from tumor and lung. (D) Relative amounts of kynurenine from tumor (left) and lung (right) between NT and JHU083 treated group. Data are from one experiment with 4-5 mice per group. **P < 0.01, ****P<0.001, Mean ± S.D. t test (B) and Mann-Whitney tests (D).
**Figure 11**

(A) 4T1 tumor lysate

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
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(B) MC38 tumor lysate

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(C) 4T1 GFP+ tumor cell

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(D) Sorted TAMs Sorted MDSCs

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<tr>
<td>Actin</td>
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(E) Kynurenine/Tryptophan

- NT
- JHU083

(F) 4T1 tumor cells

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<th>24hrs</th>
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<td>p-STAT1 (s727)</td>
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<tr>
<td>IDO</td>
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<tr>
<td>α-tubulin</td>
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(G) RAW 264.7 (macrophage cell line)

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<tr>
<td>STAT3</td>
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<td></td>
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<tr>
<td>α-tubulin</td>
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(H) D14 “Prior” to lung metastasis

- Lungs of mice without tumor
- Lungs of mice with 4T1 mammary fat pad tumor NT
- Lungs of mice with 4T1 mammary fat pad tumor JHU083

(I) TCGA normal or breast tumor data

- Glutaminolysis
- Purine synthesis pathway
- Pyrimidine synthesis pathway

- Hexosamine pathway
- Amino acid synthesis
- Coenzyme synthesis
- Nucleotide synthesis
Figure 11. Glutamine antagonism reduces IDO expression by decreasing p-STAT1/3 signaling. 
(A-D) 1x10^5 4T1 cells or 1x10^5 GFP+4T1 cells were implanted subcutaneously into mammary fat pad of BALB/cJ mice. 5x10^5 MC38 cells were implanted subcutaneously into flank of C57BL/6 mice. Tumor-bearing mice were treated with JHU083. On day 21, IDO expression in tumor lysates from (A) 4T1 or (B) MC38 tumor-bearing mice was measured by immunoblot. On day 12, (C) GFP+ tumor cells, (D) TAMs and MDSCs were sorted. Cells were lysed and IDO expression was measured by immunoblot. (E) The ratio of kynurenine to tryptophan in tumor (F) 4T1 cells were cultured in the presence or absence of DON (0.5 μM or 1 μM) and IFNγ for 6 or 24 hours. p-STAT1 (s727) and IDO expression were measured by immunoblot (left). After 6hrs with DON 1 μM treatment, Ido mRNA levels were measured by q-PCR (right). (G) RAW264.7 cells were cultured in the presence or absence of DON (0.5 μM or 1 μM) and IFNγ for 6 or 24 hours. p-STAT1 (s727), p-STAT3 (s727) and IDO were measured by immunoblot (left). After 6 hours with DON (1 μM) treatment, Ido mRNA levels were measured by q-PCR (right). (H) 4T1 tumor-bearing mice were treated with JHU083. On day 14, IDO expression within lung lysates from tumor free, and 4T1 tumor-bearing mice with or without JHU083 treatment was measured by immunoblotting. (I) Heat map visualization of p-value from pearson correlation analysis (non-log scale for calculation) using TCGA normal and breast invasive carcinoma data between the glutamine utilizing enzymes which are inhibited by DON and IDO expression (left). The graphs from each enzyme and IDO correlation data (right). *P<0.05, **P < 0.01, ****P<0.001, Mean ± S.D. Mann-Whitney t tests (E). One way ANOVA with Tukey multiple comparisons post-test (F and G).
**Figure 12.** The proposed mechanisms of how glutamine antagonism enhances anti-tumor immunity. Proposed models describing the effect of glutamine antagonism on the heterogeneous tumor micro-environment.

### Table

<table>
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<tr>
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<td>Inhibit Growth, Decreased purine, pyrimidine, protein and lipid synthesis</td>
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<tr>
<td>②</td>
<td>Decreased MDSC recruitment, Decreased C/EBPβ leading to decreased CSF3 production</td>
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<tr>
<td>③</td>
<td>Immunogenic Cell death, Increased ROS and release of DAMPs</td>
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<tr>
<td>④</td>
<td>Decreased IDO, Decreased STAT1 mediated transcription</td>
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<td><strong>Myeloid Cells</strong></td>
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<td>Increased apoptosis, Caspase-3 induced cell death</td>
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<tr>
<td>⑦</td>
<td>Enhanced M1 activation, MyD88/TRIF and TFEB dependent activation by DAMPs</td>
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Glutamine antagonist

Glutamine sufficient

Tumor cell killing mechanisms:
- **Tryp:** Trypsin
- **Kyn:** Kynurenine
- **Inhibit Growth:** Decreased purine, pyrimidine, protein and lipid synthesis
- **Decreased MDSC recruitment:** Decreased C/EBPβ leading to decreased CSF3 production
- **Immunogenic Cell death:** Increased ROS and release of DAMPs
- **Decreased IDO:** Decreased STAT1 mediated transcription

Myeloid cell apoptosis and activation mechanisms:
- **Increased apoptosis:** Caspase-3 induced cell death
- **Conversion to Inflammatory Macrophages:** Increased NF-kB activation
- **Enhanced M1 activation:** MyD88/TRIF and TFEB dependent activation by DAMPs

**Figure 12.** The proposed mechanisms of how glutamine antagonism enhances anti-tumor immunity. Proposed models describing the effect of glutamine antagonism on the heterogeneous tumor micro-environment.