Neutrophil Degranulation and Immunosuppression in Patients with GBM: Restoration of Cellular Immune Function by Targeting Arginase I

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**Translational Relevance**

Glioblastoma (GBM) remains one of the most lethal tumors known to modern medicine, and new therapeutic options are desperately needed for affected patients. Immunotherapeutic strategies have been for the most part unsuccessful in GBM, likely due to tumor-mediated suppression of cellular immune responses. The current study explored the role and functional mechanisms of peripheral myeloid cells in the suppression of cellular immunity in patients with GBM. We provide the first documentation of transferable immunosuppression associated with neutrophilic degranulation and increased circulating levels of Arginase I within peripheral blood from patients with GBM. In addition, we demonstrate that cellular immune function in patients with GBM can be restored through pharmacological inhibition of Arginase I or by supplementation with exogenous arginine. These data identify a novel pathway of GBM-mediated suppression of cellular immunity and offer a potential therapeutic window for improving anti-tumor immunity in affected patients.
Abstract

Purpose: The source of GBM-associated immunosuppression remains multifactorial. We sought to clarify and therapeutically target myeloid cell-derived peripheral immunosuppression in patients with GBM.

Experimental Design: Direct ex vivo T cell function, serum Arginase I (ArgI) levels and circulating myeloid lineage populations were compared between GBM patients and normal donors or patients with other intracranial tumors. Immunofunctional assays were performed using bulk and sorted cell populations to explore the potential transfer of myeloid cell-mediated immunosuppression and to identify a potential mechanism for these effects. ArgI-mediated immunosuppression was therapeutically targeted in vitro through pharmacological inhibition or arginine supplementation.

Results: We identified a significantly expanded population of circulating, degranulated neutrophils associated with elevated levels of serum ArgI and decreased T cell CD3ζ expression within peripheral blood from GBM patients. Sorted CD11b+ cells from patients with GBM were found to markedly suppress normal donor T cell function in co-culture, and media harvested from mitogen-stimulated GBM PBMC or GBM-associated mixed lymphoid reactions demonstrated ArgI levels that were significantly higher than controls. Critically, T cell suppression in both settings could be completely reversed through pharmacological ArgI inhibition or with arginine supplementation.

Conclusions: These data indicate that peripheral cellular immunosuppression in patients with GBM is associated with neutrophil degranulation and elevated levels of circulating ArgI, and that T cell function can be restored in these individuals by targeting ArgI. These data identify a novel pathway of GBM-mediated suppression of cellular immunity and offer a potential therapeutic window for improving anti-tumor immunity in affected patients.
**Introduction**

Treatment of patients diagnosed with glioblastoma (GBM) is one of the lasting challenges of modern medicine. Responses to standard external beam radiotherapy and chemotherapy remain dismal, providing only limited improvement in survival. Most experimental therapies have proven ineffective. The overall clinical strategy for affected individuals has not changed significantly in basic design for several decades. In spite of all best attempts, tumor recurrence is a nearly uniform phenomenon and the significant majority of patients succumb to progressive brain disease in just over one year from diagnosis (1). It is clear that new approaches for developing effective and targeted treatment options are needed for GBM patients.

To this end, immunotherapy has become a focus of recent research in GBM due to the potential for combined target specificity and sensitivity. Numerous groups have tested immunotherapeutic strategies in patients with GBM. Unfortunately, as has been seen with similar approaches in most other cancers, these efforts have been in large part unsuccessful (2). A major potential pitfall for immunotherapy in GBM is the known suppression of cellular immunity seen in affected patients, which has been well described over the past few decades. Many groups have reported on the variety of functional defects seen in the circulating pool of T cells from these individuals (3). We, and others, have documented the exceedingly rare and ultimately ineffectual T cell infiltrates found within GBM (4). In spite of these apparently local as well as global aberrations in cellular immunity, patients with GBM are **generally not systemically immunocompromised prior to the growth of their tumor**. This fact, combined with the potential for recovery of cellular immune function following surgical resection (5), has implicated a tumor-derived factor in the suppression of cell-mediated immune responses. It is therefore likely that tumor-associated immunosuppressive factors will similarly affect clinical attempts to augment anti-tumor responses. Therefore, targeting tumor-associated immunosuppression in patients with GBM will be critical for the development of meaningful immunotherapeutic strategies.

Cells of myeloid lineage have been increasingly associated with immunosuppression in a number of systems, including various forms of cancer. Myeloid-derived cells at different states of maturation have been studied as potent
inactivators of both CD4+ and CD8+ T cells (6). Populations of immature myeloid cells as well as more mature, differentiated monocytes and granulocytes have been previously shown to possess immunosuppressive abilities (7-9). Given prior observations of T cell dysfunction in GBM patients and the documentation of myeloid cells with immunosuppressive characteristics in patients with other cancers, the current study attempted to identify a myeloid-derived source of peripheral immunosuppression in GBM patients.
**Materials and Methods**

**Patient and Sample Collection:**

Peripheral blood was collected from patients undergoing neurosurgical resection of intracranial tumors (GBM, anaplastic glioma, meningioma, and pituitary tumor) at the University of Colorado Hospital with appropriate Institutional Review Board approval. Patient age and gender did not vary significantly between groups. Preoperative steroid treatment was taken into consideration; however no statistically significant differences were found between pre-operative steroid use and the presence of activated neutrophils *(reviewed in Supplementary Table 1).* Normal donor blood was collected from anonymous donors from the blood bank at the University of Colorado.

Within one hour from harvest, plasma was removed from peripheral blood samples and stored at -70°C. Peripheral blood mononuclear cells (PBMC) were purified by centrifugation over a Ficoll Histopaque (Sigma) density gradient according to the manufacturer’s protocol. PBMC were used immediately, without freezing, for T cell functional assays or staining by flow cytometry. Normal donor granulocytes used for staining by flow cytometry were collected within the flow-through fraction of the Ficoll prep; red blood cells were lysed via brief incubation in 0.84% ammonium chloride.

**T cell functional assays:**

Mitogenic stimulations were performed using bulk PBMC or isolated T cells from normal donors or patients cultured in RPMI 1640 media with 10% FBS and 1% penicillin-streptomycin. T cells were isolated using CD3 positive selection magnetic beads per manufacturer’s protocol (Miltenyi Biotec). Cells were plated at 1x10^5 cells per well in a 48 well plate with 500 μL of media. Cells were stimulated with either 1x10^5 Dynabeads (Invitrogen) or 5μg/mL Phytohemagglutinin (PHA) (Sigma) per well and incubated for 48 and 72 hours post-stimulation. Media interferon-γ (IFN-γ) levels were assayed by ELISA (Thermo Scientific) according to the manufacturer’s protocol. In functional assays where proliferation was measured by CFSE staining, isolated PBMC at a concentration of 6x10^5 cells/mL were mixed with 5 mM carboxyfluorescein succinimidyl ester (CSFE, BD Pharmingen) for 5 min and washed with media. Cells were then...
stimulated with 5μg/mL PHA and incubated for 72 hours prior to flow cytometry. For flow cytometric analysis, samples were stained with anti-CD3-APC and CD3+ T-cells were gated upon for subsequent evaluation of CFSE fluorescence. For comparative measurement of T cell proliferation as measured by CFSE dilution, the proliferation index (PI) was calculated based on the proportion of proliferating cells over total T cells.

Mixed lymphoid reactions (MLR) were carried out using bulk PBMC collected from patients and normal donors. "Modified" MLR utilized purified CD11b+ myeloid cells and CD3+ T cells from patients and normal donors, again isolated using positive bead selection. Cells from two different normal donors or a normal donor and a tumor patient were mixed at 1.0x10^5 cells/well of each cell type in 200 μL media in 96 well plates. MLR were incubated for 48 and 72 hours. Media IFN-γ levels were assayed by ELISA as described above.

**Flow Cytometry:**

Directly after isolation, 1x10^6 cells were resuspended in 200 μL FACS buffer (PBS + 20% FBS). Cells were incubated with antibodies against CD11b, CD33, CD14, HLA-DR, CD15, or CD66 (BD Biosciences) for 45 minutes at 4˚C prior to measuring expression on a FACSCalibur flow cytometer. Intracellular staining for CD3ζ was performed by resuspending CD3 stained PBMC in 100 μL of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C, washing with Perm/Wash Buffer (BD Biosciences) and staining with anti-CD3ζ (CD247) (BD Biosciences) for 45 min at 4 °C prior to measurement. Flow data was analyzed using the FlowJo software program (Treestar).

**Flow Sorting, Cytospin, and GBM Histopathological Analysis:**

CD11b+CD33lo and CD11b+CD33hi populations within PBMC from GBM patients were sorted using a FACSaria flow sorter. In parallel experiments, putative granulocytes within PBMC were isolated using magnetic bead separation by positive CD66 selection (Miltenyi Biotec). The flow-through fraction from the CD66+ selection was collected and incubated with CD11b positive selection beads to collect CD11b+CD66- monocytes. Sorted populations were spun onto Superfrost microscope slides for cytospin and histopathological analysis.
slides (Fischer) by centrifuging at 750 rpm for 2 min. Slides were stained with Wright-Giemsa stain and visualized at high power. For evaluation of actively necrotic GBM samples, formalin-fixed specimens were prepared as per standard procedures and stained with hematoxylin and eosin. Representative sections were also subject to immunohistochemical analysis for CD15 (Ventana Medical Systems) and myeloperoxidase (Dako) using standard techniques. Pathological slides were reviewed by the neuropathologist on the study (BKD).

Arginase I measurement:

Plasma samples and media from T cell functional assays described above were subject to Arginase I ELISA (Hycult Biotechnology) according to the manufacturer’s protocol. Samples were diluted 1:1 with kit dilution buffer. For evaluation of ArgI levels within necrotic material from GBM, necrotic tissue was weighed and diluted in unsupplemented RPMI media to a ratio of 60 μL/mg. The tissue was then disband and vortexed to suspend extracellular contents into the media. The resulting samples were centrifuged at 5000 rpm for 10 min to remove excess tissue and supernatants were collected. Supernatant from necrotic GBM samples were diluted over a range of 1:1 to 1:100 and used for ArgI ELISA as above.

Induction of degranulation using fMLP:

Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) (Sigma) was added to whole blood at a concentration of 1 μM and incubated at room temperature for one hour. Whole bloods without fMLP were used as controls. Following incubation, PBMC were collected using a Ficoll density gradient as described above. Bulk PBMC were used to assess T cell function through PHA stimulation and stained for flow cytometry as described previously.

For detection of dose-dependent neutrophilic suppression of T cell proliferation, neutrophils were sorted from normal donor whole blood using density centrifugation purification over a 42%/51% Percoll gradient, followed by CD66 positive bead separation. Purified neutrophils were activated with 1 μM fMLP. T cells were purified using CD3 positive separation. Cultures were then
prepared with varying T cell/neutrophil ratios as outlined in Supplementary Figure 2 and stimulated with 5 μg/mL PHA for 72 hours. BrdU was added to cultures for the final 20 hours and cells were then harvested for flow cytometric quantification of proliferation. Media Arg I levels from these cultures analyzed by ELISA as described above.

**Assays to overcome Arginase activity in vitro:**

T cell functional assays (PHA stimulation and MLR) using bulk PMBC were performed as above. Groups of samples were treated with 7.81 mg/mL L-arginine (Sigma) or 40 μM nor-NOHA (Cayman Chemicals) at the time of plating and PHA addition or cell mixing. Dose-response profiles for each compound were developed prior to testing on patient samples in order to identify the highest possible dose that did not affect baseline T cell functional response (i.e. toxicity or augmented functional response) in normal donor samples (data not shown). Cells were incubated for 48 and 72 hours and media IFN-γ levels were tested by ELISA as described above.

**Statistical Analysis:**

Data are represented as mean ± SEM. Multigroup analysis was performed using ANOVA. Differences between two variables were determined using Student’s t test. P values less than 0.05 were considered significant.
Results

Direct *ex vivo* T cells from patients with GBM are functionally suppressed *in vitro*

To confirm prior reports describing decreased proliferative responses of T cells from patients with GBM, PBMC were purified, stained with CFSE, and stimulated with PHA directly *ex vivo*. Flow cytometric analysis of stimulated T cells from patients with GBM indeed demonstrated significantly lower levels of proliferation than seen from normal donors or patients with other intracranial tumors (Figure 1A). To evaluate stimulation-induced cytokine production, levels of IFN-γ within media from PHA-stimulated PBMC cultures were assayed by ELISA. Cultures from patients with GBM generated significantly less IFN-γ at both 48 and 72 hours than did matched samples from normal donors or patients with other intracranial tumors (Figure 1B). Taken together, these results corroborate prior experimental data documenting the hyporesponsive nature of T cells in GBM patients.

PBMC and purified CD11b+ cells from GBM patients suppress normal donor T cell function

To confirm the presumptive cellular source of peripheral T cell suppression in GBM patients, we explored the possibility that GBM-associated immunosuppression could be transferred to normal donor T cells. Mixed-lymphoid reactions (MLR) were prepared using PBMC from normal donors (ND) and patients with various intracranial tumors. T cell alloresponses were confirmed by measuring IFN-γ production at 48 and 72 hours by ELISA. IFN-γ production in MLR using ND PBMC with PBMC purified from patients with pituitary tumor or meningioma demonstrated no reduction in alloresponse compared to MLR using two different ND. In contrast, T cell responses within GBM-associated MLR were markedly suppressed, producing only 20-30% of the IFN-γ as seen by ND or other intracranial tumor MLR (Figure 1C).

To further investigate the possibility that a myeloid-lineage cell within GBM PBMC was responsible for suppression of T cell activity in these assays, "modified MLR" were prepared using purified CD11b+ myeloid cells and sorted CD3+ T cells. Again, no decrease in ND T cell alloresponse was observed when cultured with CD11b+
cells from alternate ND, as measured by IFN-γ production. However, purified CD11b+ cells from GBM patients exerted a robust suppressive effect on ND T cells, resulting in similar levels of IFN-γ production as was seen in MLR using bulk PBMC (Figure 1D). Together, these results confirm that peripheral GBM-associated immunosuppression is in part associated with a CD11b+ myeloid-lineage population and that the suppressive effect can be transferred to normal donors.

**PBMC from patients with GBM harbor increased numbers of CD11b+CD33loCD14-HLA-DR- myeloid-lineage cells**

Subsequent experiments were designed to further identify the CD11b+ myeloid-derived population responsible for the transferable immunosuppressive effect. Prior studies of myeloid-related immunosuppression have identified both monocytic- and granulocytic-lineage cells within the PBMC fraction as potentially capable of suppressing T cell function (10). In order to further characterize CD11b+ cells associated with immunosuppression in GBM patients, we evaluated the PBMC fraction from GBM patients in comparison to other intracranial tumors and normal donors. The common myeloid markers CD11b and CD33, along with more specific monocytic markers CD14 and HLA-DR, were initially used to evaluate the frequency and phenotype of monocyte-lineage cells within PBMC. Evaluation of CD11b staining patterns revealed that patients with primary GBM harbor significantly increased percentages of circulating CD11b+ myeloid cells (as a proportion of total PBMC) than do patients with meningioma, pituitary tumor, or anaplastic glioma (Figure 2C). Subsequent marker analysis demonstrated that the expanded CD11b+ population within GBM patients was almost entirely composed of a distinct population expressing lower levels of CD33 (CD33lo) that segregated away from the CD14+ monocytic population expressing high levels of CD33 (CD33hi) (Figure 2A). To further confirm a non-monocytic phenotype, CD11b+CD33lo cells were shown to be negative for staining with antibodies against CD14 or HLA-DR (Figure 2B).

We next attempted to correlate the frequency of CD11b+CD33lo cells within PBMC from brain tumor patients with demographic and clinical data, including age, gender, tumor location, presence of pre-operative steroids, and imaging characteristics,
such as extent of edema, tumor location and tumor size. There was no statistically significant correlation between the presence of CD11b+CD33lo cells and any of the factors listed above. Most notably, although trending towards positive correlation, we did not observe a statistically significant correlation between presence of CD11b+CD33lo cells within PBMC from patients with intracranial tumors and pre-operative steroid therapy (p=0.10, data outlined in Supplementary Table 1) which has been previously implicated in the generation of suppressive monocytes in GBM (11).

In our evaluation of the monocyte population, we did observe a slight increase in the percentage of CD14+ monocytes within PBMCs from GBM when compared to PBMCs from normal donors. However, CD14+ monocyte frequency in GBM did not vary significantly from the percentage of CD14+ monocytes within PBMCs from patients with benign meningioma, pituitary tumor, or anaplastic glioma (Figure 2C). In addition, direct ex vivo analysis of HLA-DR expression on CD14+ monocytes from patients with GBM did not provide evidence for an HLA-DRlo population recently described within steroid-treated monocytes from GBM patients (11) (data not shown).

CD11b+CD33lo myeloid-lineage cells within GBM patient PBMC are degranulated neutrophils

As the expanded population of CD11b+CD33lo cells within PBMC from GBM patients did not appear to be of monocytic lineage, we next investigated if these cells arise from granulocytic origin. Baseline phenotypic analysis using flow cytometric scatter data demonstrated that CD11b+CD33lo cells within PBMC from GBM were smaller and more granular than characteristic monocytes seen in our prior experience (Fig 3A). In contrast, CD11b+CD33lo cells from GBM patients closely paralleled scatter characteristics exhibited by normal donor granulocytes (Figure 3A). To further verify the potential granulocytic phenotype, GBM patient PBMC were stained for the neutrophil markers CD15 and CD66; expression of these markers was similar to patterns seen on normal donor neutrophils (Figure 3B).

To provide final confirmation that CD11b+CD33lo cells within PBMC fractions from GBM patients represent neutrophils, the CD33lo and CD33hi populations were
purified for histological analysis. Initial attempts at purification using flow-sorting demonstrated the CD33lo population to be physically fragile, as membranes of sorted cells were disrupted to the point where they could not be phenotypically identified following cytospin (data not shown). In an attempt to provide a more gentle sorting process, magnetic bead separation was used to isolate CD11b+CD66+ and CD11b+CD66- populations. Subsequent histological analysis confirmed the presence of typical monocytes in the CD11b+CD66- population, while the CD11b+CD66+ population demonstrated the morphology of neutrophils (Figure 3C).

As neutrophils should normally segregate to the flow-through fraction following Ficoll density separation of whole blood, their presence within the PBMC fraction from GBM patients was somewhat puzzling. We hypothesized that the shift to the PBMC fraction could potentially arise from two sources: 1) an atypical, *de novo* granulocytic cell population generated from circulating myeloid precursors segregating with other mononuclear cells during density centrifugation, or 2) reduced density of mature circulating neutrophils, possibly secondary to degranulation, resulting in a shift to the PBMC fraction on Ficoll density gradient. As flow-sorting experiments had suggested that CD11b+CD33lo cells within PBMC from GBM patients possessed relatively weak cell membranes, we proposed to further evaluate the potential that these cells were, in fact, neutrophils in a “degranulated” state.

To first confirm the theoretical possibility that degranulation could induce a shift of neutrophils to the PBMC fraction, whole blood from normal donors was stimulated with fMLP and subject to Ficoll density centrifugation. We confirmed that fMLP-induced degranulation of normal donor neutrophils resulted in a decrease in density, corresponding with a shift to the PBMC fraction (Figure 4A), and that patterns of CD11b, CD33, CD14, and HLA-DR expression on degranulated normal donor neutrophils matched those seen in the CD11b+CD33lo population from patients with GBM (Figure 4C).

To provide additional physiological confirmation for potential *in vivo* degranulation of neutrophils in GBM patients, we attempted to document increased circulating levels of Arginase I (ArgI), a factor known to be present within neutrophilic primary granules and possess immunosuppressive activity (12). We initially confirmed that fMLP-induced
degranulation of normal donor neutrophils resulted in increased release of ArgI (data not shown). We subsequently evaluated patient plasma samples for increased levels of ArgI, and found direct ex vivo plasma ArgI levels were indeed significantly higher in patients with GBM than in normal donors or patients with other intracranial tumors (Figure 4C).

**Neutrophilic infiltrates are increased in GBM undergoing active necrosis**

Although there has been limited prior documentation of the presence of neutrophils within human GBM (13), neuropathological association of neutrophilic infiltration within necrotic tissue is well known. In prior analysis we identified limited neutrophilic infiltrates within active and infiltrative components of GBM tissue (BK DeMasters, unpublished data). However, as neurosurgeons typically provide infiltrative (i.e. “active”) tumor specimens for the purposes of pathological analysis, and necrosis in these lesions is likely a time-limited event, pathological evaluation of actively necrotic regions is often not possible. However, in isolated cases where tumors demonstrate evidence of widespread active necrosis, it is possible to evaluate the cellular infiltrate involved with the ongoing process of necrosis. We obtained several specimens of human GBM containing robust regions of active necrosis. Evaluation of H/E stained specimens from these tumors demonstrated profound neutrophilic infiltrates within the regions of acute necrosis, as would be expected with any acutely necrotic tissue (Figure 4D). Examples of GBM with more advanced coagulative necrosis and paucity of residual inflammatory cells were not examined.

**Arginase I expression correlates with T cell dysfunction in GBM patients**

ArgI has been shown to exert immunosuppressive effects through the consumption of L-arginine, a critical cofactor for sustained T cell activation due to its central role in the re-expression of the T cell co-receptor CD3ζ (14). When released into the extracellular environment, ArgI can potently and rapidly deplete extracellular L-arginine, resulting in T cell anergy and immune dysfunction. Neutrophil degranulation and subsequent release of ArgI have previously been linked to immunosuppression in renal cell carcinoma (15,16) and non-small cell lung cancer (17). We therefore
hypothesized that in vivo degranulation of neutrophils in GBM patients would result in increased levels of serum ArgI and may be a source of cellular immunosuppression.

As ArgI is known to regulate CD3ζ expression T cells, we initially explored levels of this marker on unmanipulated, direct ex vivo T cells from GBM patients. Flow cytometric analysis demonstrated that baseline CD3ζ levels were modestly lower upon circulating T cells from GBM patients than T cells from patients with pituitary tumors or meningioma (Figure 5A).

In order to demonstrate that the immunosuppressive effect of ArgI release could be recapitulated in vitro, PBMC were collected from fMLP-treated normal donor whole blood and stimulated with PHA in culture. IFN-γ levels in these cultures were compared to PHA stimulated PBMC collected from untreated normal donor whole blood. PBMC from fMLP-stimulated samples demonstrated significantly less IFN-γ production at both 48 and 72 hours compared to controls (Figure 5B). These cultures also contained elevated levels of ArgI, compared to a complete absence of ArgI present in cultures from untreated PBMC, confirming that neutrophils activated prior to purification harbor the continued capacity for release of granular contents (data not shown).

In addition, sorted normal donor T cells stimulated in the presence of increasing numbers of purified, activated neutrophils resulted in a concentration-dependent suppression of T cell proliferation (Supplementary Figure 2) confirming that neutrophil degranulation (and ArgI release, noted above) results in functional T cell suppression in vitro. To provide further evidence for an association of neutrophil degranulation with GBM-induced immunosuppression, levels of ArgI within media from in vitro GBM T cell functional cultures described previously were measured and compared to ArgI levels within media from normal donor and control patient cultures. Again, levels of ArgI were markedly elevated within media harvested from PHA-stimulated GBM PBMC, as well as within media from MLR containing GBM PBMC, when compared to normal donors or tumor controls (Figure 5C).

As mentioned above, neutrophils can be found within regions of active necrosis within GBM specimens. To demonstrate the potential for an ArgI mediated immunosuppressive effect within the tumor microenvironment, we selectively harvested
necrotic tissue during neurosurgical resection of GBM and the resulting material was subject to ArgI ELISA. Although there was significant variance between the tumors tested, all necrotic samples demonstrated ArgI levels that were manifold higher than levels observed in matched plasma samples, ranging from 171-2946 pg/mL (data not shown).

**GBM T cell function can be restored by targeting ArgI in vitro**

Initially, we confirmed that segregation of GBM patient T cells away from activated neutrophils found within PBMC resulted in a restoration of functional activation. T cells from patients with GBM, as well as normal donors, were purified from PBMC using magnetic bead separation and stimulated 1:1 with Dynabeads for 72 hours. Measurement of IFN-γ levels within culture media confirmed that functional activity of GBM T cells could be restored to levels concurrent with normal donor T cells (data not shown).

To provide pre-clinical evidence that targeting ArgI may provide benefit for restoring cellular immune function in GBM patients, we utilized several approaches to restore arginine levels within functional *in vitro* cultures. The ArgI enzymatic pathway was first targeted using the specific ArgI inhibitor nor-NOHA, which was added at a concentration of 40 μM to the media of T cell functional assays (PHA stimulation or MLR prepared as described previously). The addition of nor-NOHA to the media of functional cultures containing GBM T cells within bulk PBMC (which under normal conditions did not produce IFN-γ) resulted in a significant increase in functional response after stimulation with PHA (Figure 5D), restoring IFN-γ production to levels comparable with T cells from normal donors or patients with other intracranial tumors. A similar restitution of T cell functional response was seen in GBM-suppressed MLR in the presence of nor-NOHA (Figure 6B).

In an attempt to provide an immediately translatable mechanism for targeting neutrophil-derived ArgI activity, we explored the use of L-arginine supplementation to restore GBM T cell function *in vitro*. As seen in experiments using nor-NOHA, L-arginine supplementation significantly increased *in vitro* IFN-γ production by PHA-stimulated GBM T cells and similarly reversed suppression of normal donor T cells within MLR.
containing GBM PBMC (Figure 5D). Together, these data confirm that 1) ArgI exerts a central and reversible role in the suppression of cellular immune function in patients with GBM, and 2) that reversal of ArgI-mediated effects through either pharmacological inhibition or addition of exogenous L-Arg can restore GBM T cell function to levels equivalent with normal controls.
Discussion

In spite of a long history of study outlining GBM-associated effects on cellular immunity, there has been little understanding of the underlying factors responsible for the observed suppression. Our analysis has confirmed that T cells from GBM patients demonstrate minimal proliferation and IFN-γ production upon activation directly \textit{ex vivo}. However, to our knowledge, the current study represents the first documentation of increased numbers of degranulated neutrophils within the peripheral circulation of GBM patients. This phenomenon has been previously described in several other human cancers (16,17,22), perhaps first outlined by Rodriguez et al. in their analysis of patients with renal cell cancer. We observed similar expression patterns of myeloid-lineage markers within the cell population of interest and confirmed the neutrophilic phenotype through histopathological analysis. As in the renal cell cancer cohort, we observed increased ArgI in plasma samples from GBM patients. We have further expanded upon the hypothetical effect of increased ArgI release \textit{in vivo} by confirming a concomitant decrease in surface CD3ζ expression on T cells from GBM patients analyzed directly \textit{ex vivo}. Importantly, we provide herein the first evidence that neutrophil-mediated suppression of T cell function in cancer patients can be reversed \textit{in vitro} using either selective pharmacological inhibition of ArgI or, more simply, through the addition of exogenous L-arginine. In addition, the identification of increased frequency of neutrophils and massively elevated ArgI levels within actively necrotic GBM specimens offers not only potential insight into the ontological source of degranulated neutrophils in these patients, but also a potential mechanism through which cellular immunity may be disrupted within the tumor microenvironment.

Myeloid lineage cells with immunosuppressive properties, recently categorized as myeloid-derived suppressor cells (MDSC), have been previously associated with cellular immunosuppression in a number of disease states (7). In animals, phenotypic classification of MDSC has been fairly straightforward and is well correlated with functional suppression of T cell activity. More recently, increasing data in humans has confirmed the presence of cells with functional characteristics of MDSC, although the phenotypic nomenclature remains somewhat less clear (6-9). For the most part, MDSC can be subdivided into two phenotypic populations having either monocytic or
granulocytic characteristics, identified in humans as CD14+HLA-DRneg or CD14negHLA-DRneg respectively. Both subsets can suppress T cell function although multiple lineage-specific mechanisms for this effect have been proposed (10,18,19). Prior studies have explored immunosuppressive qualities of monocytic populations within GBM patients (10,11). Gustafson et al. recently identified an expanded population of MDSC within steroid-treated patients with GBM, phenotypically defined as CD14+HLA-DRlo/neg (11). We were unable to document a similar monocytic population within our cohort and could not identify a difference in monocytic expression patterns associated with steroid treatment. A potential explanation for this discrepancy could derive from differing methods for tissue handling; most notably, all analyses in our study were performed with fresh (1-4 hours post-resection) PBMC and frozen samples were never utilized. It has been our experience that monocytic expression patterns can change rapidly with freeze-thaw cycles, and neutrophils from patients or normal donors do not survive the freeze-thaw process.

Though it is likely that monocytic populations within GBM patients may contribute to the observed immunosuppressive effect, our studies have shown a strong correlation between the presence of degranulated neutrophils and T cell dysfunction. The ability of normal granulocytes to suppress T cell function has been previously described (21) and has also been linked to immunosuppression in non-small cell lung, pancreatic, colon and breast cancer (17,22). Populations of granulocytic MDSC have also been described in renal cell carcinoma (15) and non-small cell lung cancer (23). Whether described as MDSC or normal neutrophils, the mechanism by which granulocytic cells induce immunosuppression is commonly linked to the release of ArgI into the extracellular environment. While monocytic MDSC-derived immunosuppression has also been linked to ArgI expression, human monocytic cells tend to deplete extracellular L-arginine via increased CAT2B expression and intracellular transport. Transported L-arginine is subsequently metabolized by intracellular ArgI (a biological characteristic demonstrated by alternatively activated (M2) macrophages) (24). The fact that ArgI is found at increased extracellular levels within the plasma of GBM patients suggests that the enzyme is released from the expressing cell, consistent with a granulocytic mechanism of ArgI-mediated immunoregulation.
Neutrophils have been well characterized in their role for promoting inflammation and combating infections after tissue damage has occurred (25). With these biological characteristics in mind, it is perhaps no surprise that neutrophils would be attracted to the tumor microenvironment. This phenomenon is particularly likely within GBM, as this fast-growing tumor harbors necrosis as one of its defining pathological characteristics. In regards to potential sources of active neutrophilic recruitment in GBM, previous studies have shown that pseudopalisading cells surrounding regions of necrosis within GBM release the cytokine IL-8, a factor with strong trophic effects upon neutrophils (26, 27). However, given complex cellular and biological characteristics of the tumor microenvironment, a large range of additional candidate mechanisms that may induce neutrophilic recruitment and induction of degranulation mandate significant further experimentation. Ongoing studies in our group are exploring potential GBM-specific factors that would explain the observed neutrophilic changes in affected patients.

In the current study, we have identified two possible mechanisms through which ArgI-mediated T cell dysfunction may be reversed in vitro. Perhaps most importantly, we have confirmed that the simple supplementation of extracellular L-arginine to T cell functional assays can reverse the immunosuppressive phenotype. Ironically, L-arginine supplementation has been previously utilized within non-cancer clinical settings. On an initially empiric basis, oral arginine supplementation was explored and found to demonstrate efficacy for improving immune function in patients suffering major trauma or undergoing extensive surgical procedures (28). It was subsequently confirmed that ArgI is transiently found at increased levels in these patients (29,30), supporting the clinical utility for dietary L-arginine supplementation in the reversal of immunosuppression. These clinical results encourage parallel translation to cancer patients. Oral L-arginine supplementation is clinically attractive due to low cost, ease of delivery, and negligible toxicity. Based upon our recent data, we have initiated a pilot clinical trial exploring the utility of oral L-arginine supplementation for restoring endogenous cellular immunity that is, in part, suppressed by activated neutrophils in newly-diagnosed GBM patients. Although augmentation of T cell function by targeting ArgI in vivo may not, in isolation, confer significant clinical benefit in regards to tumor clearance, we predict that reversal of ArgI-mediated suppression of cellular
immunity may offer a critical therapeutic adjuvant for the development of effective immunotherapy in patients with GBM.
References:

Figure Legends

Figure 1: GBM patient T cell function is suppressed; myeloid cells from GBM patients can transfer suppression to normal donors. (A) Representative (left panel) and averaged (right panel) flow cytometric analysis of gated CD3+ CFSE-stained T cells from GBM patients (n=5) when compared to normal donors (ND, n=6) or T cells from patients with pituitary tumors (PIT, n=6) (p=0.006). (B) ELISA measurement of IFN-γ within culture media after PBMC stimulation with PHA in patients with GBM (n=10), meningioma (MEN, n=5), pituitary tumor (n=9) or ND (n=20) (p<0.0001). (C) Measurement of IFN-γ production in MLR using bulk PBMC (ND n=5, MEN n=5, PIT n=8, GBM n=10) (p<0.0001). (D) IFN-γ production in "modified" MLR using isolated CD3+ T cells (T) from ND and isolated CD11b+ myeloid cells (M) from alternate ND (n=8) or GBM patients (n=9) at both 48 (p<0.0001) and 72 hrs (p<0.0001).

Figure 2: GBM patients harbor an expanded population of circulating CD11b+CD33lo cells that appear within the PBMC fraction. (A) PBMC from normal donors and patients with meningioma or GBM were stained for the myeloid markers CD11b and CD33. (B) Gated CD11b+CD33hi and CD11b+CD33lo cells within GBM PBMC were stained for the prototypic monocytic markers CD14 and HLA-DR. (C) Analysis of average CD11b+CD33hi and CD11b+CD33lo population frequency between all patients sampled (outlined in Supplementary Table 1) (p= 0.016).

Figure 3: CD11b+CD33lo cells within GBM PBMC express granulocyte markers and demonstrate histological characteristics of neutrophils. (A) Representative forward and side scatter analysis of flow cytometry data from ND and GBM PBMC as well as the flow-through fraction after Ficoll centrifugation of ND blood; circular gates represent cells with scatter characteristics of granulocytes. (B) ND granulocytes, collected from the flow-through fraction after Ficoll centrifugation, and gated CD11b+CD33lo cells from GBM PBMC stained with the granulocytic markers CD15 and CD66. Corresponding isotypes found in Supplemental Figure 1. (C) Wright-Giemsa stain of sorted CD11b+CD66+ and CD11b+CD66- cells from GBM PBMC.
**Figure 4:** CD11b+CD33lo cells within GBM PBMC are degranulated neutrophils. (A) Degranulation was induced in normal donor whole blood using fMLP; PBMC were collected from stimulated blood and matched unstimulated controls and subject to flow cytometry; **circular gates represent cells with scatter characteristics of granulocytes.** (B) Flow cytometric analysis of gated CD11b+CD33lo cells within fMLP-degranulated normal donor PBMC, stained with CD14, HLA-DR, CD15, and CD66. **Corresponding isotypes found in Supplemental Figure 1.** (C) Arginase I ELISA analysis of plasma samples from GBM patients (n=6) when compared to ND (n=10) or patients with meningioma (MEN, n=14) or metastatic tumors (MET, n=5) (p<0.0001). (D) Low (i)- and high (ii)-magnification images as well as immunohistochemical staining for CD15 (iii) and myeloperoxidase (iv) from a representative sample of GBM undergoing active necrosis; arrows mark an area of active necrosis.

**Figure 5:** Arginase I levels correlate with GBM T cell dysfunction *in vitro* and *in vivo*. (A) Representative (**left panel**) and averaged (**right panel**) flow cytometric analysis of intracellular CD3ζ expression in direct *ex vivo* T cells from PIT (blue, n=6), MEN (green, n=6), and GBM (orange, n=5) patients compared to isotype (black) (p=0.024). (B) ELISA measurement of PHA-induced IFN-γ production by normal donor PBMC, following neutrophil degranulation by fMLP in whole blood prior to PBMC purification, when compared to matched PBMC from unstimulated blood at 48 (p=0.0007) and 72 hrs (p=0.015). (C) Comparative ELISA-based measurements of ArgI levels in immunofunctional assays (PHA stimulation and MLR) using bulk PBMC from normal donors (n=4) or patients with meningioma (n=4) or GBM (n=5) (p<0.0001). (D) The ArgI inhibitor nor-NOHA (n=4) or supplemental L-arginine (n=5) were added to **(left panel)** PHA stimulated **bulk PBMC** or **(right panel)** MLR developed from the aforementioned groups. IFN-γ production was measured by ELISA at 48 hours. (p<0.0001 in each case).

**Supplemental Table 1:** Demographics of patients used to analyze the relationship between pre-operative steroids and presence of CD11b+CD33lo suppressive cells. All patients undergoing neurosurgical resection were treated with peri-
operative steroids 1-2 hours prior to blood acquisition. Patients noted to have received pre-operative steroids (Y) were treated with dexamethasone for varying time periods at varying doses prior to the day of surgery. Normal donor demographics are not reported, as blood was collected from anonymous donors at the blood bank of the University of Colorado.

Supplemental Figure 1: Flow cytometry isotypes. (A) Isotypes corresponding to CD66 and CD15 plots in Figure 3B. (B) Isotypes corresponding to CD14, HLA-DR, CD66 and CD15 plots in Figure 4B.

Supplemental Figure 2: Activated Neutrophils suppress T cell proliferation in a dose dependent manner which correlates with the presence of ArgI. (A) Purified neutrophils from normal donors (n=5) were activated with fMLP and mixed at varying concentrations with sorted T cells. Cultures were stimulated with PHA for 72 hours and proliferation was measured by BrdU incorporation in gated CD3+ populations (p<0.05). (B) Media from cultures was collected and ArgI was measured by ELISA (p<0.0001).
FIGURE 1

A.

B.

C.

D.
FIGURE 2

A. Normal donor
- CD33hi: 2.94
- CD33lo: 0.44

B. Meningioma
- CD33hi: 3.03
- CD33lo: 4.54

C. GBM
- CD33hi: 2.94
- CD33lo: 13.37

Legend:
- CD33
- CD11b
- CD14
- HLA-DR

Graph C shows the percentage of PBMC (Peripheral Blood Mononuclear Cells) for different conditions:
- ND (Normal Donor)
- PIT
- APG
- MEN
- GBM

* indicates a significant difference.
FIGURE 3
Clinical Cancer Research

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