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

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Abstract

**Purpose:** The source of glioblastoma (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 patients with GBM and normal donors or patients with other intracranial tumors. Immunofunctional assays were conducted 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 pharmacologic 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 patients with GBM. Sorted CD11b+ cells from patients with GBM were found to markedly suppress normal donor T-cell function in coculture, and media harvested from mitogen-stimulated GBM peripheral blood mononuclear cell (PBMC) or GBM-associated mixed lymphoid reactions showed ArgI levels that were significantly higher than controls. Critically, T-cell suppression in both settings could be completely reversed through pharmacologic 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 antitumor 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 1 year from diagnosis (1). It is clear that new approaches for developing effective and targeted treatment options are needed for patients with GBM.

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 antitumor 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 stages 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 patients with GBM and the documentation of myeloid cells with immunosuppressive characteristics in patients with other cancers, the current study is attempted to identify a myeloid-derived source of peripheral immunosuppression in patients with GBM.

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 preoperative steroid use and the presence of activated neutrophils (reviewed in Supplementary Table S1). Normal donor (ND) blood was collected from anonymous donors from the blood bank at the University of Colorado.

Within 1 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 density gradient (Sigma) according to the manufacturer’s protocol. PBMCs were used immediately, without freezing, for T-cell functional assays or staining by flow cytometry. ND 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 conducted using bulk PBMCs or isolated T cells from ND or patients cultured in RPMI-1640 media with 10% FBS and 1% penicillin–streptomycin. T cells were isolated using CD3⁺ selection magnetic beads per manufacturer’s protocol (Miltenyi Biotec). Cells were plated at 1 × 10⁵ cells per well in a 48-well plate with 500 µL of media. The cells were stimulated with either 1 × 10⁵ Dynabeads (Invitrogen) or 5 µg/mL phytohemagglutinin (PHA; Sigma) per well and incubated for 48 and 72 hours poststimulation. Media IFN-γ levels were assayed by ELISA (Thermo Scientific) according to the manufacturer’s protocol. In functional assays where proliferation was measured by carboxyfluorescein succinimidyl ester (CFSE) staining, isolated PBMCs at a concentration of 6 × 10⁵ cells/mL were mixed with 5 mmol/L CFSE (BD Pharmaningen) for 5 minutes and washed with media. Cells were then stimulated with 5 µg/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 on the basis of proportion of proliferating cells over total T cells.

Mixed lymphoid reactions (MLR) were carried out using bulk PBMCs collected from patients and ND. *Modified* MLR used purified CD11b⁺ myeloid cells and CD3⁺ T cells from patients and ND, again isolated using positive bead selection. Cells from 2 different ND or a ND and a tumor patient were mixed at 1.0 × 10⁵ cells per 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, 1 × 10⁶ cells were resuspended in 200 µL FACS buffer (PBS + 20% FBS). Cells were incubated

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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 (ArgI) within peripheral blood from patients with GBM. In addition, we show that cellular immune function in patients with GBM can be restored through pharmacologic inhibition of ArgI 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 antitumor immunity in affected patients.
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 FACS Calibur flow cytometer. Intracellular staining for CD3**+** was carried out by resuspending CD3-stained PBMCs in 100 μl of Cytotox/Cytoperp solution (BD Biosciences) for 20 minutes at 4°C, washed with Perm/Wash Buffer (BD Biosciences), and stained with anti-CD3**+** (CD247; Santa Cruz Biotechnology) for 45 minutes at 4°C prior to measurement. Flow data were analyzed using the FlowJo Software program (Treestar).

**Flow-sorting, cytospin, and GBM histopathologic analysis**

CD11b**+** CD33lo and CD11b**+** CD33hi populations within PBMCs from patients with GBM were sorted using a FACSAria flow sorter. In parallel experiments, putative granulocytes within PBMCs 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**+** selection beads to collect CD11b**+** CD66**−** monocytes. Sorted populations were spun onto Superfrost microscope slides (Fischer) by centrifuging at 750 rpm for 2 minutes. 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. Pathologic slides were reviewed by the neuropathologist on the study (B.K. DeMasters).

**Arginase I measurement**

Plasma samples and media from T-cell functional assays described above were subject to Arginase I (ArgI) 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 1:1 to 1:100. 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 μmol/L and incubated at room temperature for 1 hour. Whole bloods without fMLP were used as controls. Following incubation, PBMCs were collected using a Ficoll density gradient as described above. Bulk PBMCs 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 ND whole blood using density centrifugation purification over a 42%/51% Percoll gradient, followed by CD66**+** bead separation. Purified neutrophils were activated with 1 μmol/L fMLP. T cells were purified using CD3**+** separation. Cultures were then prepared with varying T-cell to neutrophil ratios as outlined in Supplementary Fig. S2 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 ArgI levels from these cultures were analyzed by ELISA as described above.

**Assays to overcome arginase activity in vitro**

T-cell functional assays (PHA stimulation and MLR) using bulk PBMCs were conducted as above. Groups of samples were treated with 7.81 mg/mL L-arginine (Sigma) or 40 μmol/L 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 to identify the highest possible dose that did not affect baseline T-cell functional response (i.e., toxicity or augmented functional response) in ND 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 conducted using ANOVA. Differences between 2 variables were determined using the Student 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 and PBMCs were purified, stained with CFSE, and stimulated with PHA directly ex vivo. Flow cytometric analysis of stimulated T cells from patients with GBM indeed showed significantly lower levels of proliferation than seen from ND or patients with other intracranial tumors (Fig. 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 ND or patients with other intracranial tumors (Fig. 1B). Taken together, these results corroborate prior experimental data documenting the hypo-responsive nature of T cells in patients with GBM.

**PBMCs and purified CD11b**+** cells from patients with GBM suppress normal donor T-cell function**

To confirm the presumptive cellular source of peripheral T-cell suppression in patients with GBM, we explored the possibility that GBM-associated immunosuppression could be transferred to ND T cells. MLR were prepared using
PBMCs from 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 PBMCs with PBMCs purified from patients with pituitary tumor or meningioma showed no reduction in alloresponse compared with MLR using 2 different ND. In contrast, T-cell responses within GBM-associated MLR were markedly suppressed, producing only 20% to 30% of the IFN-γ as seen by ND or other intracranial tumor MLR (Fig. 1C).

To further investigate the possibility that a myeloid lineage cell within GBM PBMCs 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 patients with GBM exerted a robust suppressive effect on ND T cells, resulting in similar levels of IFN-γ production as was seen in MLR using bulk PBMCs (Fig. 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 ND.

PBMCs 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). To further characterize CD11b+ cells associated with immunosuppression in patients with GBM, we evaluated the PBMC fraction from patients with GBM in comparison to other intracranial tumors and ND. 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 monocytic-lineage cells within PBMCs. 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 PBMCs) than do patients with meningioma, pituitary tumor, or anaplastic glioma (Fig. 2C). Subsequent marker analysis showed that the expanded CD11b+ population within patients with GBM 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; Fig. 2A). To further confirm a nonmonocytic phenotype, CD11b+CD33lo cells were shown to be negative for staining with antibodies against CD14 or HLA-DR (Fig. 2B).

We next attempted to correlate the frequency of CD11b+CD33lo cells within PBMCs from patients with brain tumor with demographic and clinical data, including age, gender, tumor location, presence of preoperative 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 toward positive correlation, we did not observe a statistically significant correlation between presence of CD11b+CD33lo cells within PBMCs from patients with intracranial tumors and preoperative steroid therapy (P = 0.10, data outlined in Supplementary Table S1) 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 with PBMCs from ND. 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 (Fig. 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 patients with GBM (ref. 11; data not shown).

**CD11b^+CD33lo myeloid lineage cells within GBM patient PBMCs are degranulated neutrophils**

As the expanded population of CD11b^+CD33lo cells within PBMCs from patients with GBM 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 showed that CD11b^+CD33lo cells within PBMCs from GBM were smaller and more granular than characteristic monocytes seen in our prior experience (Fig. 3A). In contrast, CD11b^+CD33lo cells from patients with GBM closely paralleled scatter characteristics exhibited by ND granulocytes (Fig. 3A). To further verify the potential granulocytic phenotype, GBM patient PBMCs were stained for the neutrophil markers CD15 and CD66; expression of these markers was similar to patterns seen on ND neutrophils (Fig. 3B).

To provide final confirmation that CD11b^+CD33lo cells within PBMC fractions from patients with GBM represent neutrophils, the CD33lo and CD33hi populations were purified for histologic analysis. Initial attempts at purification using flow sorting showed that the CD33lo population was 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 histologic analysis confirmed the presence of typical monocytes in the CD11b^+CD66^- population, whereas the CD11b^+CD66^+ population showed the morphology of neutrophils (Fig. 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 patients with GBM was somewhat puzzling. We hypothesized that the shift to the PBMC fraction could potentially arise from 2 sources: (i) an atypical, de novo granulocytic cell population generated from circulating myeloid precursors segregating with other mononuclear cells during density centrifugation, or (ii) 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 PBMCs from patients with GBM 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 ND was stimulated with fMLP and subject to Ficoll density centrifugation. We confirmed that fMLP-induced degranulation of ND neutrophils resulted in a decrease in density, corresponding with a shift to the PBMC fraction (Fig. 4A), and that patterns of CD11b, CD33, CD14, and HLA-DR expression on degranulated ND neutrophils matched those seen in the CD11b^+CD33lo population from patients with GBM (Fig. 4C).

To provide additional physiologic confirmation for potential in vivo degranulation of neutrophils in patients...
with GBM, we attempted to document increased circulating levels of ArgI, a factor known to be present within neutrophilic primary granules and possess immunosuppressive activity (12). We initially confirmed that fMLP-induced degranulation of ND neutrophils resulted in increased release of ArgI (data not shown). We subsequently evaluated patient plasma samples for increased levels of ArgI, and found that direct ex vivo plasma ArgI levels were indeed significantly higher in patients with GBM than in ND or patients with other intracranial tumors (Fig. 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), neuropathologic 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 pathologic analysis, and necrosis in these lesions is likely a time-limited event, pathologic evaluation of actively necrotic regions is often not possible. However, in isolated cases where tumors show 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 showed profound neutrophilic infiltrates within the regions of acute necrosis, as would be expected with any acutely necrotic tissue (Fig. 4D). Examples of GBM with more advanced coagulative necrosis and paucity of residual inflammatory cells were not examined.

ArgI expression correlates with T-cell dysfunction in patients with GBM

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 coreceptor CD3ζ (14). When released into the extracellular environment, ArgI can potently and rapidly deplete extracellular L-arginine, resulting in T-cell energy 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 patients with GBM would result in increased

Figure 3. CD11b+CD33lo cells within GBM PBMCs express granulocyte markers and show histologic characteristics of neutrophils. A, representative forward (FSC) and side scatter (SSC) analysis of flow cytometric data from ND and GBM PBMCs 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 PBMCs stained with the granulocytic markers CD15 and CD66. Corresponding isotypes found in Supplementary Fig. S1. C, Wright–Giemsa stain of sorted CD11b+CD66+ and CD11b+CD66– cells from GBM PBMCs.
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 patients with GBM. Flow cytometric analysis showed that baseline CD3ζ levels were modestly lower upon circulating T cells from patients with GBM than T cells from patients with pituitary tumors or meningioma (Fig. 5A).

To show that the immunosuppressive effect of ArgI release could be recapitulated in vitro, PBMCs were collected from fMLP-stimulated samples showed significantly less IFN-γ production at both 48 and 72 hours compared with controls (Fig. 5B). These cultures also contained elevated levels of ArgI, compared with a complete absence of ArgI present in cultures from untreated PBMCs, confirming that neutrophils activated prior to purification harbor the continued capacity for release of granular contents (data not shown).

In addition, sorted ND T cells stimulated in the presence of increasing numbers of purified, activated neutrophils...
further evidence for an association of neutrophil degranulation (and ArgI release, noted above) with GBM-induced immunosuppression, levels of ArgI within media from in vitro GBM T-cell functional cultures described previously were measured and compared with ArgI levels within media from ND and control patient cultures. Again, levels of ArgI were markedly elevated within media harvested from PHA-stimulated GBM PBMCs, as well as within media from MLR containing GBM PBMCs, when compared with ND or tumor controls (Fig. 5C).

As mentioned above, neutrophils can be found within regions of active necrosis within GBM specimens. To show 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 showed ArgI levels that were manifold higher than levels observed in matched plasma samples, ranging from 171 to 2,946 pg/mL (data not shown).

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

Initially, we confirmed that segregation of T cells from patients with GBM away from activated neutrophils found within PBMCs resulted in a restoration of functional activation. T cells from patients with GBM, as well as ND, were purified from PBMCs 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 ND T cells (data not shown).

To provide preclinical evidence that targeting ArgI may provide benefit for restoring cellular immune function in patients with GBM, we used several approaches to restore ArgI 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 μmol/L 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 PBMCs (which under normal conditions did not produce IFN-γ) resulted in a significant increase in functional response after stimulation with PHA (Fig. 5D), restoring IFN-γ production to levels comparable with T cells from ND 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 (Fig. 5D).

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 ND T cells within MLR containing GBM PBMCs (Fig. 5D). Together, these data confirm that (i) ArgI exerts a central and reversible role in the suppression of cellular immune...
function in patients with GBM, and (ii) that reversal of Arg-mediated effects through either pharmacologic inhibition or addition of exogenous L-arginine 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 patients with GBM show minimal proliferation and IFN-γ production upon activation directly ex vivo. However, to our knowledge, the current study represents the first documentation of increased numbers of degranulated neutrophils within the peripheral circulation of patients with GBM. This phenomenon has been previously described in several other human cancers (16, 17, 18), perhaps first outlined by Rodriguez and colleagues 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 histopathologic analysis. As in the renal cell cancer cohort, we observed increased ArgI in plasma samples from patients with GBM. We have further expanded upon the hypothetical effect of increased ArgI release in vivo by confirming a concomitant decrease in surface CD3ζ expression on T cells from patients with GBM analyzed directly ex vivo. Importantly, we provide herein the first evidence that neutrophil-mediated suppression of T-cell function in patients with cancer can be reversed in vitro using either selective pharmacologic 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 ontologic 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 2 phenotypic populations having either monocytic or granulocytic characteristics, identified in humans as CD14+HLA-DRlo/neg or CD14negHLA-DRnog, respectively. Both subsets can suppress T-cell function although multiple lineage-specific mechanisms for this effect have been proposed (10, 19, 20). Prior studies have explored immunosuppressive qualities of monocytic populations within patients with GBM (11, 21). Gustafson and colleagues 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 conducted with fresh (1–4 hours postresection) PBMCs and frozen samples were never used. It has been our experience that monocytic expression patterns can change rapidly with freeze–thaw cycles, and neutrophils from patients or ND do not survive the freeze–thaw process.

Though it is likely that monocytic populations within patients with GBM 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 (22) and has also been linked to immunosuppression in non–small cell lung, pancreatic, colon and breast cancer (17, 18). 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 monocytes cells tend to deplete extracellular L-arginine via increased CAT2B expression and intracellular transport. Transformed T cells are subsequently metabolized by intracellular ArgI (a biologic characteristic shown by alternatively activated (M2) macrophages; ref. 24). The fact that ArgI is found at increased extracellular levels within the plasma of patients with GBM 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 biologic 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 pathologic 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 interleukin (IL)-8, a factor with strong trophic effects upon neutrophils (26, 27). However, given complex cellular and biologic 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
GM-specific factors that would explain the observed neutrophilic changes in affected patients.

In the current study, we have identified 2 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 extra-cellular l-arginine to T-cell functional assays can reverse the immunosuppressive phenotype. Ironically, l-arginine supplementation has been previously used within noncancer clinical settings. On an initially empiric basis, oral arginine supplementation was explored and found to show 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 patients with cancer. Oral l-arginine supplementation is clinically attractive due to low cost, ease of delivery, and negligible toxicity. On the basis of 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 patients with GBM. 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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