Flow cytometry and in vitro analysis of human glioma–associated macrophages

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Abstract

Object—To date, glioma immunotherapy has been focused mostly on stimulating antitumor peripheral lymphocyte responses; however, some data suggest that microglia and/or macrophages (not lymphocytes) are the predominant inflammatory cells infiltrating gliomas. To study this hypothesis further, the authors analyzed inflammatory cell infiltrates in fresh human malignant glioma specimens and primary cultures.

Methods—Single-cell suspensions from fresh operative malignant glioma specimens, obtained by stereotactic localization, were analyzed for CD11b and CD45 by using flow cytometry. A comparison was made with peripheral blood mononuclear cells. In a subset of patients, a more detailed flow cytometry analysis of Class I and II major histocompatibility complex, B7-1, B7-2, CD11c, and CD14 expression was performed. Macrophage-like cells in primary glioma cultures were similarly assessed.

Results—Operative samples were obtained from 9 newly diagnosed malignant gliomas. The mean percent of $CD45^+/CD11b^-$ cells (lymphocytes) was 2.48% (range 0.65–5.50%); $CD45^{dim}/CD11b^+$ cells (microglia), 1.65% (range 0.37–3.92%); and $CD45^{bright}/CD11b^+$ (monocytes/ macrophages), 6.25% (range 1.56–15.3%). More detailed fluorescence-activated cell sorting suggested that macrophage-like cells expressed Class I and II major histocompatibility complex, B7-2, and CD11c but not CD14 or B7-1. Primary human glioma cultures contained significant numbers of macrophage-like ($CD45^{bright}/CD11b^+$) cells, but these cells were lost with successive passages. These cells maintained the immunomarker profiles of macrophage-like cells from fresh specimens only if they were cultured in serum-free media.

Conclusions—The $CD45^+/CD11b^+$ cells are the predominant inflammatory cell infiltrating human gliomas. Of this type, the $CD45^{bright}/CD11b^+$ cells, a phenotype compatible with circulating macrophages in rodent models, and not microglia, are the most common. Their immunomarker profile is compatible with an immature antigen-presenting cell. They are present in

primary glioma cultures but are lost in successive passages. Their role is enigmatic, and they may prove an important target for future glioma immunotherapy studies.

Keywords

glioma; immunotherapy; lymphocyte; macrophage; microglia

Malignant gliomas are the most common primary tumors of the CNS. Despite significant advances in surgery, radiation therapy, and chemotherapy over the past 3 decades, the median patient survival remains < 1 year from lesion diagnosis.[26,27,31] This finding has prompted the investigation of novel therapeutic strategies, including immunotherapy. So far, glioma immunotherapy efforts largely have been focused on stimulating antitumor lymphocyte responses. For example, several types of glioma vaccines using various strategies have recently been developed and tested.[28,30,36,46,48,50] These vaccines have occasionally led to measurable antiglioma lymphocyte responses, but clinical responses have been less impressive.[42,47] This result in part may reflect the nature of the small pilot and Phase I studies that have been designed to evaluate the feasibility and safety of the therapy but not its efficacy. Nonetheless, it is known that the microenvironment within gliomas is immunosuppressive because of the expression of several factors (transforming growth factor–β2, prostaglandin E2, interleukin-6, and Fas ligand) that downregulate immune responses.[13,24,34] Local glioma immunosuppression has not been adequately addressed in most vaccine trials.

The local immune microenvironment within gliomas is dependent on both glioma cells and other cell types such as infiltrating inflammatory cells. It is noteworthy that the lymphocyte's role in the glioma microenvironment may be limited. Some data suggest that microglia (the brain's resident antigen-presenting cells) and/or macrophages—not lymphocytes—are the predominant inflammatory cells infiltrating gliomas.[20,21,23,38] There is evidence from rodent models that glioma-associated microglia do not participate in effective antiglioma immune responses and may actually secrete immunosuppressive factors such as interleukin-10 and Fas ligand.[6,23,43,52] Targeting these cells may be an important immunotherapeutic strategy.

It has been reported that inflammatory cell subpopulations can be characterized in normal and inflamed rat brain on the basis of CD45 (leukocyte common antigen) and CD11b (complement receptor 3) 2-color flow cytometry.[41] Four populations can be distinguished: CD45$^-$/CD11b$^-$ (noninflammatory cells), CD45$^+$/CD11b$^-$ (lymphocytes), CD45^{dim}/CD11b$^+$ (microglia), and CD45^{bright}/CD11b$^+$ (monocytes/macrophages). Distinguishing resident microglia from infiltrating systemic macrophages by relative CD45 expression is based on bone marrow transplant experiments. Animals were irradiated to ablate bone marrow and then received allogeneic bone marrow transplants. Brain inflammation was subsequently induced with increases in both CD45^{dim}/CD11b$^+$ and CD45^{bright}/CD11b$^+$ infiltrates. Haplotyping showed that the CD45^{dim} population retained the parental haplotype (suggesting they were resident microglia), whereas the CD45^{bright} population had the transplant haplotype (suggesting they were infiltrating systemic macrophages).[41]

The CD45/CD11b flow cytometry has been used to differentiate lymphocytes, peripheral monocytes/macrophages, and microglia in rodent models of experimental allergic encephalitis.[40,41] More recently, Badie and colleagues[3,7] have used this technique to analyze inflammatory cell infiltrates in several rat glioma models. They found marked microglial (CD45^{dim}/CD11b$^+$) infiltration into both tumor and adjacent cortex. In many cases, this progression was also associated with significant lymphocytic infiltration. As similar studies have not been conducted in human gliomas, we used CD45/CD11b flow cytometry to

quantitate the number of lymphocytes, microglia, and monocytes/macrophages in human glioma specimens. We also performed more detailed flow cytometry characterization of surface immunomarker expression in a subset of glioma specimens. Finally, we sought to establish a method of culturing human glioma–derived microglia to facilitate future studies of glioma-microglia interactions.

Methods

Obtaining Human Glioma Specimens

Fresh surgical specimens from patients with untreated, newly diagnosed primary gliomas were collected at the time of craniotomy and processed immediately. Using an intraoperative image guidance system based on preoperative Gd-enhanced MR imaging (Stealth, Medtronic), samples were taken from enhancing tumor (9 samples) and overlying "normal" cortex (5 samples, when cortical resection was indicated). All specimens from enhancing areas were histopathologically confirmed as GBM (World Health Organization Grade IV astrocytoma). Specimens were obtained in accordance with a University of California, San Francisco, Committee on Human Research–approved tissue banking protocol at the Brain Tumor Research Center.

Preparation of Single-Cell Suspensions and Primary Cultures

Single-cell suspensions and primary cultures were obtained from fresh operative specimens as previously described.[33] Briefly, specimens were minced and washed repeatedly with PBS, and obvious hematoma was removed. The resulting slurry was subjected to partial enzyme digestion (DNAse, pronase, and collagenase, Roche Bioscience and GibcoBRL), passed through 100-µm nylon mesh, and spun on a density gradient (Ficoll-Paque, Amersham Biosciences). Cells at the interface were collected and washed twice in PBS. The resulting single-cell suspensions were either used directly for flow cytometry or plated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin G (Cell Culture Facility, University of California, San Francisco) or in serum-free adaptive immunotherapy medium (AIM V, GibcoBRL) and cultured at 37°C, 5% CO_2.

Isolation of PBMCs

Peripheral blood mononuclear cells were obtained from healthy donors via leukophoresis tubing. Samples were spun on a density gradient (Ficoll-Paque Plus, Amersham Biosciences). Cells at the interface were harvested, washed once in PBS, and used immediately for flow cytometry analysis.

Flow Cytometry

Single-cell suspensions from operative specimens were washed once in PBS, resuspended in PBS, counted on a hemocytometer with trypan blue staining, and divided into 10^6-cell aliquots for flow cytometry. Cells in these aliquots were washed again in PBS, resuspended in 100 µl of PBS with 1% human AB serum, and incubated at room temperature for 10 minutes for Fc-receptor blocking. Thereafter, 10 µl of antihuman CD45-FITC (eBioscience) and 10 µl of antihuman CD11b-PE (eBioscience) were added to each sample, and the samples were incubated at room temperature in the dark for 15 minutes. Similar staining was performed with isotype-matched control antibodies (eBioscience). Cells were washed in PBS, resuspended in 100 µl of PBS with 5 µl of 7-AAD (BD Pharmingen), and incubated at room temperature in the dark for 10 minutes. An additional 300 µl of PBS was added to each sample, and the samples were immediately read on a flow cytometer on the FL-1, -2, and -3 parameters with the aid of CellQuestPro software (both Becton Dickinson). An analysis was

performed using the Flow-Jo software (TreeStar, Inc.). Dot plots for CD45 versus CD11b expression were generated after excluding debris by forward- and side-scatter gating and 7-AAD$^+$ cells by leaving out dead cells.

For a subset of samples, more detailed flow cytometry analysis was performed. In addition to the CD45/CD11b staining described above, samples were stained with 10 μl of antihuman HLA-ABC–FITC (BD Pharmingen) and 10 μl of antihuman CD11c-PE (eBioscience), 10 μl of antihuman HLA-DR–FITC (Caltag Laboratories), 10 μl of antihuman B7-1–FITC (eBioscience, 10 μl of antihuman B7-2–PE (eBioscience), and 10 μl of antihuman CD14-FITC (Miltenyi Biotec). Staining (including 7-AAD staining) and analysis proceeded as above.

Analysis of Primary Human Glioma Cultures

Primary (Passage 1) and early-passage (Passages 2–4) human GBM cultures at 80% confluence were harvested by scraping after an incubation for 15 minutes in 5 mM EDTA at 37°C. They were washed, stained for CD11b, CD14, and CD45 as above, and analyzed immediately by flow cytometry. The CD11b$^+$ cells were purified from primary human glioma cultures by harvesting in EDTA as above and then separating the CD11b$^+$ cells by using antihuman CD11b-coated microbeads according to the manufacturer's instructions (Miltenyi Biotec).

Results

Fresh operative specimens were obtained from 9 patients harboring GBMs. Based on intraoperative image guidance (Stealth), samples were harvested from enhancing tumor or cortex overlying tumor (Fig. 1). As expected, flow cytometric analysis of CD45/CD11b expression patterns in fresh enhancing human GBM specimens revealed 4 distinct populations (CD45$^-$/CD11b$^-$, CD45$^+$/CD11b$^-$, CD45^{dim}/CD11b$^+$, and CD45^{bright}/CD11b$^+$) corresponding to the noninflammatory cells, lymphocytes, microglia, and macrophages identified in rodent models (Fig. 2A). Note, however, that the macrophage-like population (CD45^{bright}/CD11b$^+$) was the most common inflammatory cell identified (mean [± SD] 6.25 ± 5.30% of viable cells, range 1.56–15.3%). Lymphocytes (CD45$^+$/CD11b$^-$: mean 2.48 ± 1.64% of viable cells, range 0.65–5.50%) and microglia-like cells (CD45^{dim}/CD11b$^+$: mean 1.65 ± 1.28% of viable cells, range 0.37–3.92%) were less common (Fig. 2B). Macrophage-like cells were significantly more common than lymphocytes (p < 0.05, Tukey 2-tailed analysis of variance), whereas microglia-like cells were not. This finding was relatively surprising, as microglia and lymphocytes have been reported to be the most common inflammatory cells infiltrating rat glioma models.[7]

Similarly, 4 distinct populations could be identified in "normal" cortex samples based on CD45/CD11b staining and flow cytometry (Fig. 3A). Macrophage-like cells (CD45^{bright}/CD11b$^+$) were the most common infiltrating inflammatory cells (mean 0.49 ± 0.50% of viable cells, range 0.11–1.30%), followed by microglia-like cells (CD-45^{dim}/CD11b$^+$: mean 0.32 ± 0.30% of viable cells, range 0.03–0.82%) and lymphocytes (CD45$^+$/CD11b$^-$: mean 0.22 ± 0.16% of viable cells, range 0.06–0.43%; Fig. 3B). Interestingly, the amount of infiltrating inflammatory cells in the cortex was an order of magnitude lower than in enhancing tumor (Fig. 2). This finding was also relatively surprising, as previous reports on rat glioma models have suggested that "normal" cortex in glioma-bearing animals has increased inflammatory cell infiltrates compared with truly normal cortex in non–glioma-bearing animals.[7]

It is possible that some of the inflammatory cells identified in our flow cytometry study represent contaminating PBMC blood retained on and within the tumor specimen. However,

CD45/CD11b staining of and flow cytometry for human glioma specimens reveal a distinct pattern when compared with PBMCs (Fig. 4). As expected, PBMCs do not include significant numbers of CD45$^-$/CD11b$^-$ (noninflammatory) or CD45^{dim}/CD11b$^+$ (microglia) cells. Furthermore, far more CD45$^+$/CD11b$^-$ cells (lymphocytes) are present than CD45^{bright}/CD11b$^+$ cells (monocytes/macrophages). The ratio of CD45$^+$/CD11b$^-$ cells (lymphocytes) to CD45$^+$/CD11b$^+$ cells (monocytes/macrophages/microglia) is significantly higher in healthy donor PBMCs (mean ± standard deviation 3.71 ± 1.01, 5 patients) than in glioma specimens (0.48 ± 0.54, 9 patients; $p < 0.001$, Student 2-tailed t-test). These data strongly suggest that the inflammatory cells identified in our flow cytometry study represent a distinct population of glioma-associated cells and not simply contaminating PBMCs.

A more extensive flow cytometry study was performed on a subset of glioma samples (Fig. 5). Human leukocyte antigen–ABC (Class I MHC) consistently demonstrated a biphasic expression pattern in which most cells (presumably tumor cells) showed moderate expression and a sub-set (presumably inflammatory cells) showed high expression. Most cells were HLA-DR (Class II MHC) negative, but a subset was HLA-DR positive. This subset roughly corresponded numerically to the monocyte/macrophage and microglial cells identified by CD45/CD11b flow cytometry. Furthermore, HLA-DR$^+$ cells were also positive for CD11b (data not shown). The CD11c and B7-2 cells were moderately positive in a subset of cells roughly corresponding numerically to the monocyte/macrophage and microglial cells identified by CD45/CD11b flow cytometry. The B7-1 and CD14 staining was negative.

To facilitate further testing of monocyte/macrophage/microglia interactions with human gliomas, we sought techniques to establish cultures of glioma-derived monocyte/ macrophage/microglia cells. A number of techniques for establishing microglial cultures from brain specimens have been reported.[12,17,19,49] Several of these procedures (including separation on a density gradient, rotary shaking, and serum starvation) have been attempted with human glioma single-cell suspensions and primary cultures but did not yield significant numbers of CD11b$^+$ cells (data not shown). However, we noted that primary cultures (Passage 1) from human glioma specimens often contained significant numbers of CD11b$^+$ cells (Fig. 6A). This macrophage-like population was lost with subsequent passages but could be separated into a pure population of CD11b$^+$ cells by sorting with magnetic beads (Fig. 6B). Attempts to sort pure populations of CD11b$^+$ cells from fresh (uncultured) human glioma single-cell suspensions were unsuccessful, possibly because of the presence of contaminating debris (data not shown). It was apparent that different culture conditions could considerably modify immunomarker expression in macrophage-like cells in primary human glioma cultures. Primary cultures plated in standard medium (DMEM/F12 supplemented with 10% fetal bovine serum) showed reduced numbers of cells expressing CD11b or CD11c but increased CD14$^+$ and CD45$^+$ cells compared with primary cultures plated in serum-free AIM V medium (Fig. 6C). The immunomarker expression pattern in cells cultured in serum-free medium more closely resembled the pattern seen in infiltrating inflammatory cells in fresh gliomas specimens (Fig. 5).

Discussion

As expected, based on previous reports in rodent glioma models,[4,7,23] monocyte-lineage cells formed the most prominent group of inflammatory cells infiltrating human gliomas. Unexpectedly, we found that these cells were predominantly CD45^{bright}/CD11b$^+$. This phenotype corresponds to infiltrating systemic macrophages, not resident microglia (CD45^{dim}/CD11b$^+$), in rodent models.[7,41] However, the validity of distinguishing microglia and macrophages based on the CD45^{dim}/CD11b$^+$ or CD45^{bright}/CD11b$^+$ phenotype has not been established in humans. Therefore, we believe that these data suggest that systemic

macrophages (not resident microglia) are the most common inflammatory cell infiltrating human gliomas, although we recognize that we cannot be dogmatic in this assertion. In any event, monocyte-lineage cells are clearly more common than lymphocytes in human gliomas. This finding contrasts with data on rat 9L gliosarcoma and C6 glioma (the most common glioma models used for testing immunotherapies) in which lymphocytes can account for up to one-third of viable cells.[7]

Similarly, we were somewhat surprised that inflammatory cell infiltrates were not more numerous in "normal" cortex overlying malignant gliomas given that previous studies have suggested that "normal" cortex in rats bearing glioma or gliosarcoma xenografts demonstrate increased inflammatory cell infiltrates compared with cortex in non–tumor-bearing animals.[7] The reason for this difference is not entirely clear. It is possible that commonly used rat glioma or gliosarcoma models are inherently immunogenic.[35] This explanation would be in keeping with the increase in lymphocytic infiltration into tumors in these models[7] compared with our results in fresh, human surgical specimens. That being the case, the increased inflammatory cell infiltrate in neighboring cortex in these models, compared with human specimens, may simply reflect an increased immune response against the tumor resulting in more inflammatory cells homing to the CNS.

In rodent models in which CD45/CD11b flow cytometry is used to analyze inflammatory cells infiltrating the CNS, animals are killed and perfused with saline prior to harvesting brains for analysis.[7,41] This step is performed to reduce contaminating the PBMCs retained within CNS vasculature, which might confound analysis of parenchymal inflammatory cells. For obvious reasons, this step cannot be performed prior to harvesting glioma specimens from human patients. It is possible that some of the inflammatory cells we identified using CD45/CD11b flow cytometry represent contaminating PBMCs were retained with the tumor specimens. However, CD45$^+$/CD11b$^-$ cells are more common than CD45$^+$/CD11b$^+$ cells in PBMCs but much less common in fresh glioma specimens (Fig. 4). This finding strongly suggests that we identified a distinct population of glioma-associated inflammatory cells, and not simply contaminating PBMCs.

The more detailed flow cytometry study used to examine surface immunomolecules was performed in a small subset of patients and thus must be interpreted with caution (Fig. 5). Nevertheless, some interesting observations can be made. As expected, most cells (presumably tumor cells) had modest Class I MHC expression and no Class II MHC. This finding is in keeping with previous reports on MHC expression in human gliomas.[2,25,32,39] A small subset of cells was positive for Class II MHC and highly positive for Class I MHC. This population corresponds numerically to the monocyte-lineage (CD45$^+$/CD11b$^+$) cells and appears to be double positive for Class II MHC and CD11b$^+$ cells (data not shown). Similarly, a subset of cells (corresponding numerically to CD45$^+$/CD11b$^+$ cells) was moderately positive for CD11c and the T cell costimulatory molecule B7-2. Note that B7-1 (another T cell costimulatory molecule) and CD14 (bacterial lipopolysaccharide receptor) were absent on all cells.

Taken together, these findings suggest that the macrophage-like cells infiltrating human gliomas are Class I MHC$^+$/Class II MHC$^+$/CD11b$^+$/CD11c$^+$/CD14$^-$/CD45$^+$/B7-1$^-$/B7-2$^+$. This phenotype is compatible with immature macrophages as more mature, activated macrophages would be expected to have increased CD14 and B7-1 expression and decreased B7-2 expression; most lymphocytes would be Class II MHC$^-$ and CD11c$^-$. [15,16,37] The absence of CD14 expression is particularly striking given that its expression is nearly universally increased in macrophages and microglia in CNS inflammatory disorders. [8–11,18,22,51] The combination of marked infiltration of monocyte-lineage cells and CD14 downregulation has not been reported in other CNS disorders. This combination may have

important implications for proposed therapeutic strategies utilizing lipopolysaccharide or cytidine phosphate guanosine–containing oligonucleotides to stimulate antiglioma immune responses[14,45] as CD14 is the receptor for both of these agents. However, we are reluctant to characterize these apparently immature glioma-associated macrophages as "inactive" based on the present study. We have not examined other aspects of their immune function such as phagocytic ability, antigen-presenting ability, and cytokine secretion. It is possible that glioma-conditioned macrophages and dendritic cells have an actively immunosuppressive phenotype characterized by the secretion of immunosuppressive cytokines, ability to induce apoptosis or anergy in activated T cells, and/or stimulation of immunosuppressive T regulatory cells.[1,5,29,43,44] Further study of glioma-associated macrophage and microglia function is required to clarify these issues.

The data in this paper are descriptive. It would be desirable to develop model systems in which glioma/glioma-associated macrophage interactions can be tested more rigorously. Most monocyte-lineage cells infiltrating gliomas in this study had a macrophage-like (CD45^{bright}/CD11b$^+$) as opposed to a microglia-like (CD45^{dim}/CD11b$^+$) phenotype. This result suggests that examining interactions between peripheral blood monocytes (macrophage precursors) and glioma cells could be highly relevant. The coculture of human glioma cells with peripheral blood monocytes is a simple model that may be adequate to test these interactions. This coculture model has several advantages, including the existence of many immortalized human glioma cell lines and the relative ease with which monocytes can be isolated from PBMCs.

Note, however, that there are also distinct disadvantages to using monocyte/glioma interactions as a model for glioma interactions with glioma-associated macrophages in situ. First, the system described above is allogeneic. This feature may not be relevant when examining pure glioma/monocyte interactions but could significantly cloud results if allogeneic lymphocytes are added to the system to examine the functional effects of glioma/ monocyte interactions such as antigen presentation. Using an autologous system of patient PBMCs and explanted tumor cultures may avoid this particular drawback. Still, infiltrating macrophage-like cells may be irreversibly altered by interactions with glioma cells in situ in ways that cannot be replicated by short-term coculture of glioma cells and peripheral blood monocytes. Furthermore, the distinction between peripheral macrophages and resident microglia on the basis of the CD45^{bright}/CD11b$^+$ and CD45^{dim}/CD11b$^+$ phenotype has not been established in humans.[41] It is possible that the CD45^{bright}/CD11b$^+$ cells infiltrating gliomas are microglia after all. In that case, using peripheral blood monocytes to model microglial reactions may only be partially accurate.

For all of these reasons, it would be desirable to culture glioma-infiltrating macrophage-like cells so that autologous glioma explant/glioma-associated macrophage/lymphocyte coculture systems could be developed. We applied several standard techniques for establishing microglial cultures from normal human brain tissue (rotary shaking, serum starvation, or separation on a density gradient) to fresh glioma specimens and cultures, but we were unable to generate microglial cultures given the overwhelming presence of tumor cells (data not shown). Similarly, we were unable to isolate microglia cultures by sorting fresh glioma single-cell suspension with CD11b-magnetic beads (data not shown), perhaps because of the presence of considerable debris.

We did find that primary human glioma cultures (Passage 1) contained significant numbers of CD11b$^+$ (macrophage-like) cells (Fig. 6). This population disappeared with successive passages. These cells could be easily sorted into a pure viable CD11b$^+$ population by using magnetic beads, but this population did not survive as an independent culture. Although our present inability to establish pure glioma-associated macrophage cultures is somewhat

limiting, the presence of these cells in primary explant cultures suggests that the cultures could be used as the basis for an autologous model of glioma/glioma-associated macrophage/lymphocyte interactions. Efforts are ongoing to establish pure glioma-associated macrophage cultures by changing the sorting techniques and/or adding cytokines or glioma-conditioned medium. Our finding that inflammatory cells in primary cultures established in serum-free medium have immunomarker profiles more similar to those in fresh surgical specimens suggests that the presence of glioma-associated macrophages may be an important aspect of establishing these in vitro model systems.

Documenting that primary glioma explant cultures contain significant numbers of inflammatory cells as well as tumor cells is important for several reasons. It has been reported that primary glioma cultures have immunological characteristics that are lost in successive passages.[2] Although this change has been believed to reflect a loss of innate glioma characteristics with prolonged exposure to in vitro conditions, our data instead suggest that it may reflect a loss of associated inflammatory cells. Furthermore, several investigators have suggested tailoring glioma therapy based on screening primary glioma cultures for chemosensitivity and/or genetic profile.[31] These efforts are likely to be unrewarding unless primary culture heterogeneity is taken into account.

Conclusions

Glioma immunotherapy to date largely has been focused on stimulating antiglioma responses by peripheral lymphocytes without any attempts to address local immunosuppressive conditions. Comprehensive efforts to analyze the role of glioma-associated macrophage/microglia cells have been even less common. In this paper, we presented data suggesting that monocyte-lineage cells (not lymphocytes) are the most common inflammatory cells infiltrating human gliomas. These cells have characteristics compatible with infiltrating systemic macrophages, not resident microglia. Preliminary data indicate that they have an immunologically immature phenotype, but more investigation is required. Coculturing human glioma cells and peripheral blood monocytes may be a simple and relevant model for performing these studies given that glioma-infiltrating cells resemble peripheral macrophages. Nonetheless, it would still be desirable to isolate glioma-associated macrophages for further investigation. We have not been able to establish pure glioma-associated macrophage cultures, but we have found that primary human glioma cultures contain significant numbers of macrophage-like cells that are lost with successive passages. Therefore, primary glioma cultures themselves may represent a useful system for studying glioma-macrophage interactions, and attempts are ongoing to purify macrophages from these cultures. The cellular heterogeneity of primary glioma cultures has important implications for other studies based on primary explant cultures.

Acknowledgments

Abbreviations used in this paper

DMEM/F12	Dulbecco modified Eagle medium and Hams F12 medium
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
HLA	human leukocyte antigen
MHC	major histocompatibility complex

| **PBMC** | peripheral blood mononuclear cell |
PBS	phosphate-buffered saline
PE	phycoerythrin
7-AAD	7-aminoactinomycin D

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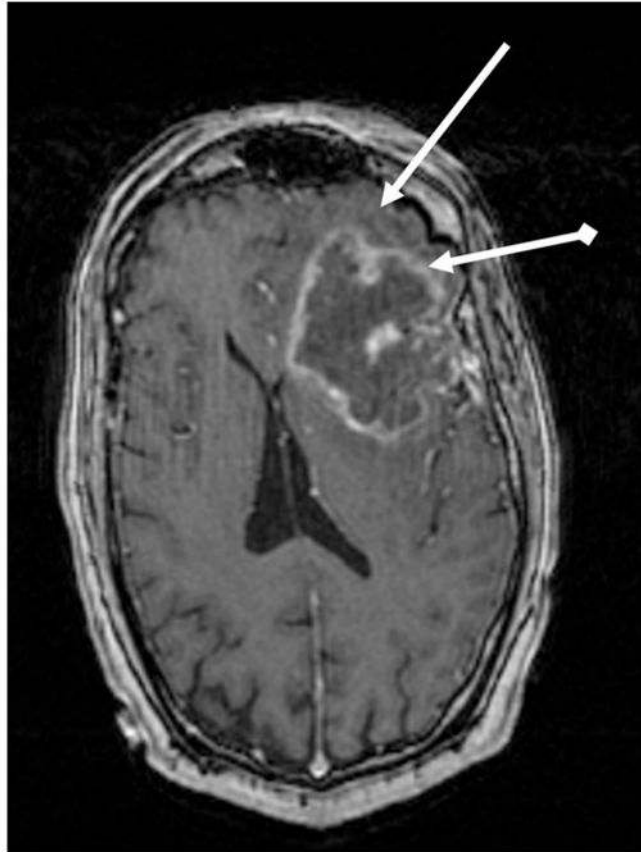
Fig. 1.
Representative T1-weighted Gd-enhanced MR image of a GBM demonstrating areas of
resected overlying cortex *(plain arrow)* and enhancing tumor *(arrow with diamond)*.
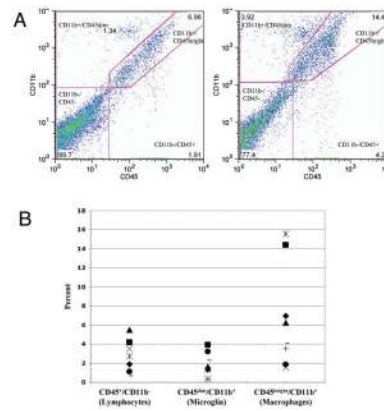
Fig. 2.
A: Two representative dot plots showing CD45 and CD11b staining for enhancing GBM specimens by flow cytometry. Each *dot* represents a single stained cell. Four distinct populations can be seen: CD45$^-$/CD11b$^-$, CD45$^+$/CD11b$^-$, CD45^{dim}/CD11b$^+$, and CD45^{bright}/CD11b$^+$. Double negative gates are based on isotype-matched controls (not shown). B: Summary scatter plot showing the percentage of total viable cells that were CD45$^+$/CD11b$^-$, CD45^{dim}/CD11b$^+$, and CD45^{bright}/CD11b$^+$. Each *shape* represents a tumor specimen taken from a specific patient.
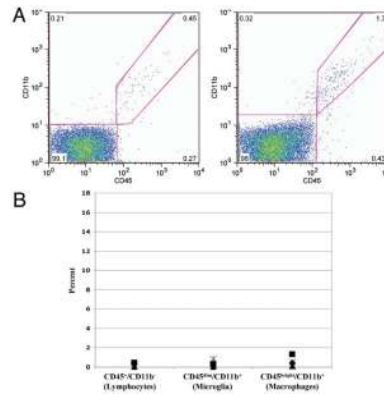
Fig. 3.
A: Two representative dot plots showing CD45 and CD11b staining for cortex overlying tumor by flow cytometry. Similar types of CD45 and CD11b positive and negative cells can be seen as for enhancing tumor, but the absolute number of inflammatory cells is much lower. B: Summary scatter plot demonstrating the percentage of viable cells that are CD45$^+$/CD11b$^-$, CD45^{dim}/CD11b$^+$, and CD45^{bright}/CD11b$^+$. Each *shape* represents a tumor specimen taken from a specific patient.
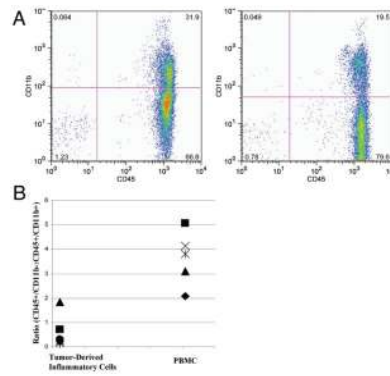
Fig. 4.
A: Two representative dot plots showing CD45 and CD11b staining for normal PBMC samples by flow cytometry. Only CD45$^+$/CD11b$^-$ and CD45^{bright}/CD11b$^+$ cells are visible. B: Ratio of CD45$^+$/CD11b$^-$ to CD45$^+$/CD11b$^+$ cells for PBMCs and enhancing tumor specimens (GBMs). Marked differences are seen, suggesting that the CD45$^+$ and CD11b$^+$ cells in tumor specimens do not simply represent residual PBMCs. Each *shape* represents a tumor specimen taken from a specific patient.
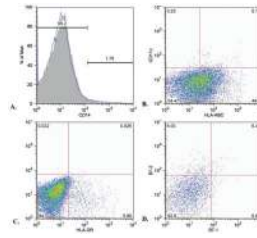
Fig. 5.
Representative flow cytometry histogram (A) and dot plots (B–D) of more detailed immunomarker profiles of single-cell suspensions of fresh glioma specimens performed in a subset of patients. Expression of CD14 (A, essentially absent on all cells). Class I MHC (HLA-ABC) and CD11c expression (B). Note a biphasic Class I MHC expression (mostly moderately positive with a strongly positive subset) and limited CD11c expression. Class II MHC (HLA-DR) expression (C); a subset of cells is positive. Expression of B7-1 and B7-2 (D). Expression of the former is very limited, but B7-2 expression is seen as a subset of cells. Note that the percentage of Class I MHC^{high}, Class II MHC^{+}, and B7-2^{+} cells roughly corresponds numerically to the number of CD45^{bright}/CD11b^{+} cells for this sample (6.24%, data not shown)
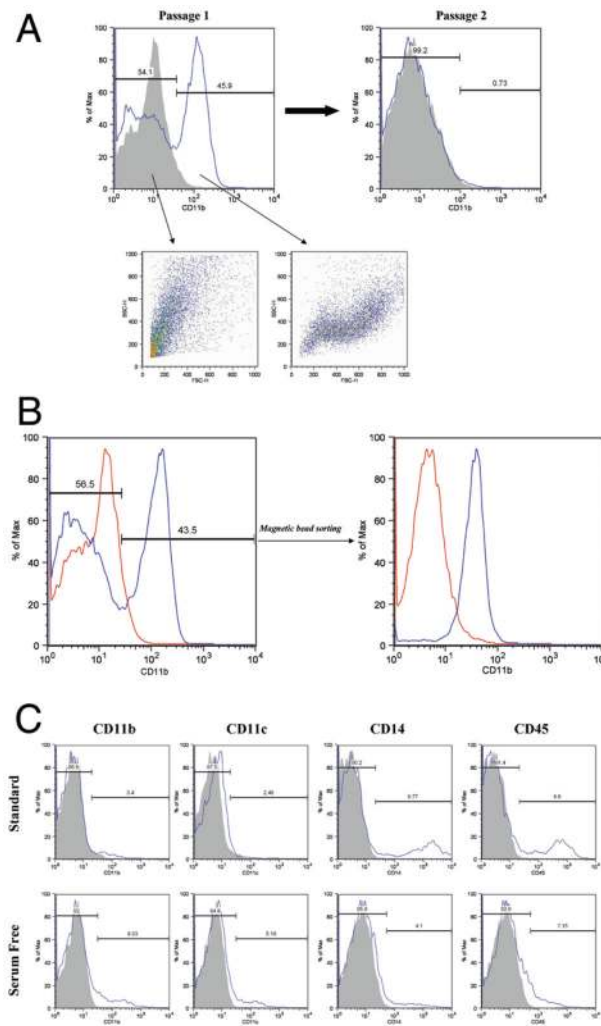
Fig. 6.
A: Histogram (Passage 1) showing CD11b expression in a primary human glioma culture. Distinct CD11b⁻ and CD11b⁺ populations can be seen that have distinct forward scatter (FSC) and side scatter (SSC) properties. Note that the CD11b⁺ population is lost by Passage 2. B: Histograms showing purification of CD11b⁺ cells from glioma primary culture by sorting with anti–CD11b-coated microbeads. C: Histograms showing percentage of inflammatory cell marker-positive cells seen in primary glioma cultures established from a single specimen in the presence or absence of serum.