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Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma + vaccination model

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ABSTRACT

In rats bearing an intracranial T9 glioma, immunization with tumor antigens induces myeloid suppressor cells, which express neutrophil (His48) and monocyte (CD11bc) markers, to infiltrate the tumors. The His48$^+$/CD11bc$^+$ cells were not derived from CNS microglia but were hematogenous; suppressed multiple T cell effector functions; and are myeloid-derived suppressor cells (MDSC). The glioma-infiltrating MDSC expressed arginase I, iNOS, indoleamine 2,3-dioxygenase and TGF-β; however, inhibitor/blocking studies demonstrated that NO production was the primary mechanism of suppression which induced T cell apoptosis. These findings suggest that neuro-immunomodulation by MDSC in rat gliomas maybe mediated by a pathway requiring NO production.

1. Introduction

Glioblastomas are grade IV astrocytic neoplasms derived from the glial lineage; represent ~50% of primary central nervous system tumors; and are essentially incurable with a median survival of approximately 18 months after primary diagnosis (Surawicz et al., 1998). Current standard treatment is surgical debulking followed by concomitant temozolomide chemotherapy and radiotherapy which has shown to extend median survival and additional 2.5 months (Stupp et al, 2009).

Many brain tumor researchers believe that the development of a glioma vaccine, which can activate glioma-specific T cells, would be an effective compliment to current treatment and could be used to destroy residual tumor cells and prevent recurrence. Immunization strategies to include tumor cells that have been genetically altered to secrete immune enhancing cytokines; dendritic cells that have been manipulated to present tumor antigens; and active immunization with recombinant cancer peptides (reviewed by Luptrawan et al., 2008; Okada and Pollack, 2004; Selznick et al., 2008). However, because of the immunosuppressive environment of the brain tumor, these approaches have met with little success. Malignant gliomas produce soluble factors such as transforming growth factor (TGF)-β, prostaglandins and interleukin (IL)-10 which are part of the immunosuppressive tumor environment (reviewed in Gomez and Kruse, 2006). Immunomodulating cells which also suppress T cells function are found to infiltrate gliomas, such as T regulatory cells, macrophages and microglial (reviewed in Sonabend et al., 2008; Watters et al., 2005). More recently, we and others have identified immuno-regulatory myeloid cells that infiltrate experimental rodent brain tumors (Prins et al., 2002; Thomas et al., 2008). These tumor infiltrating myeloid cells are phenotypically double positive for granulocyte and monocyte markers, His48/CD11bc (rat) or Gr1/CD11b (mouse). It has yet to be determined if these tumor-associated, regulatory cells represent a population of: myeloid-derived suppressor cells (MDSC); immature-regulatory dendritic cells; or microglial cells (Cools et al., 2007; Nagaraj and Gabrilovich, 2007; Watters et al., 2005).

The generation of MDSC has been generally reported in non-glial, murine tumor models. However, there are also studies of MDSC in other pathological settings such as experimental autoimmune encephalomyelitis, graft-vs-host disease, bacterial infections and severe trauma models in which down-modulation of T cell activity is a common feature (al Ramadi et al., 1991; Bobe et al., 1999; Makarenkova et al., 2006; Zhu et al., 2007). Human MDSC have been recently described in cancers such as melanoma and in renal cell carcinoma (Filipazzi et al., 2007; Ko et al., 2009; Zea et al., 2005).

There are multiple mechanisms by which MDSC exert their regulatory effects on activated T cells some of which include the catabolism of essential amino acids such as tryptophan or arginine by indoleamine 2,3-dioxygenase (IDO) or arginase 1, respectively; production of nitric oxide (NO); or by the production of immunosuppressive cytokines such as TGF-β (Mazzoni et al., 2002; Mellor and Munn, 2004; Rodriguez and Ochoa, 2008; Xiang et al., 2008). Immature dendritic cells and regulatory microglia have been reported to down-modulate T cell activity by the production of TGF-β (Gandhi et al., 2007; Watters et al., 2005).

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In the T9 + vac paradigm, s.c. vaccination of animals with an established, intracranial (i.c.) T9 glioma results in the trafficking of T cells to the tumor site. Immunization also induces His48$^+$/CD11bc$^+$ immature myeloid cells to infiltrate the intracerebral tumors (Prins et al., 2002). In this report, we identify the His48$^+$/CD11bc$^+$ cells as rat MDSC in the T9 + vac model based upon phenotype, origin and ability to suppress multiple T cell effector functions. Moreover, we demonstrate that NO production by the MDSC mediate T cell inhibition and induces T cell apoptosis. We believe that neuro-immunoregulatory MDSC represent another method in which gliomas can evade tumor reactive T cells and MDSC may play a role in brain tumor-related immunosuppression.

2. Materials and methods

2.1. Animals, cell lines and tumor cell culture

Inbred female Fischer 344 rats weighing 100–120 g were obtained from the National Cancer Institute (Fredrick, MD). Animals were housed in a climate controlled, AAALAC approved, vivarium and were provided free access to rat chow and water. All experimental animal procedures were approved by members of the Institutional Animal Care and Use Committee. The T9 glioma and MadB106 mammary adenocarcinoma were chemically induced in female Fischer rats (Barlozzari et al., 1985; Benda et al., 1971). T9 and MadB106 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and non-essential amino acids (Invitrogen) as adherent monolayers at 37 °C, passed biweekly with trypsin in the absence of antibiotics. Tumor cells were routinely screened for mycoplasma contamination (MycoTect, Invitrogen).

2.2. Tumor implantation and vaccination

Intracranial implantation of T9 glioma cells and vaccination with irradiated T9 cells were performed as previously described (Prins et al., 2002). Briefly, 5×10^5 T9 cells in 5 μl of PBS were slowly injected intracranially at a depth of 3.5 mm, 4 mm to the right of the sagital suture and 1 mm posterior to the coronal suture and 5 days later rats were vaccinated s.c. with 5×10^6 irradiated (50 Gy) T9 cells in 100 μl of PBS. In this model, vaccinated rats become moribund in ~14 days and non-vaccinated animals become moribund in ~25 days.

2.3. Depletion His48$^+$ or CD11bc$^+$ cells and purification of MDSC from the glioma infiltrate

Tumors from moribund T9 + vac rats were mechanically forced through a 70 μM mesh to generate a single cell suspension. Cells were washed; layered onto Histopaque 1.119 (Sigma, St. Louis, MO); and centrifuged for 30 min at 700×*g*. The interface containing mononuclear cells and granulocytes was collected and residual erythrocytes were removed by hypotonic lysis. Viable tumor infiltrating leukocytes (TIL) were enumerated on a hemacytometer. His48$^+$ or CD11bc$^+$ cells were depleted from the TIL by positive selection using immunomagnetic columns and microbeads (Miltenyi Biotec, Auburn, CA) according to supplied protocol. In brief, TIL were incubated with biotinylated anti-His48 or CD11bc mAbs (1×10^6 cells/0.25 μg) for 20 min; washed with PBS; incubated with anti-biotin microbeads; and positively selected on a LS MACS separation column. Glioma infiltrate depleted of His48$^+$ or CD11bc$^+$ cells were used in subsequent proliferation assays. The positively selected CD11bc$^+$ cells from the tumor infiltrate that were retained in the magnetic column were purged with PBS and used in add-back, T cell/MDSC co-culture experiments.

2.4. Antibodies and flow cytometry

Antibodies were obtained from BD Biosciences (San Diego, CA) unless otherwise noted. For the depletion or purification of MSC, biotinylated His48 or CD11bc mAbs (Cedarlane Laboratories, Burlington, NC) were used. Anti-CD3 conjugated to fluorescein isothiocyanate (FITC); CD4 conjugated to Cy-Chrome; anti-CD8 conjugated to phycoerythrin; and an anti-bromodeoxyuridine (BrdU) conjugated to allophycocyanin mAbs were used in T cell proliferation studies. For phenotypic analysis of the immature myeloid cells, anti-CD11b conjugated to phycoerythrin and a biotinylated anti-His48 mAbs were used in conjunction with streptavidin–peridinin chlorophyll protein. FITC was used as a third color and Abs were conjugated to FITC and include anti-: CD2, CD3, CD4, CD8, CD11b, CD45, CD45RA, CD54, CD86, CD90, CD161, RP-3 (granulocytes), MHC class I (RT1A) and MHC class II (RT1B). Cell surface staining and FACS analysis of TIL from T9 + vac animals have been previously described (Prins et al., 2002). Briefly, 1×10^6 cells were stained in a volume of 50 μl of 5% fetal bovine serum/PBS containing a cocktail of 3 different mAbs for 30 min on ice. Cells that were double positive His48^{high} and CD11bc$^+$ were identified as MSC and were gated upon for subsequent phenotypic analysis.

2.5. T cell proliferation assays

Tumor infiltrating leukocytes depleted of His48$^+$ or CD11bc$^+$ cells or total TIL were seeded at a concentration of 1×10^6 cells/ml in triplicate and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, HEPES (10 mM) and antibiotics/antimycotic (RPMI medium). Lymphocytes were stimulated with 5 μg/ml immobilized anti-CD3 and 0.5 μg/ml soluble anti-CD28 (clones G4.18 and JJ319, respectively, BD Biosciences) for 3 days and pulsed with BrdU (10 μM, BD Biosciences) for the last 24 h. Cells were collected and stained with anti-CD3, CD4 and CD8 mAbs cocktail. Cells were then fixed, permeabilized and stained with an anti-BrdU mAb using a BrdU flow detection kit (BD Biosciences). Lymphocytes were then analyzed by flow cytometry on a FACSCanto cytometer (BD Biosciences) with initial gating on the CD3$^+$/CD4$^+$ or CD3$^+$/CD8$^+$ populations.

In add-back assays, splenic lymphocytes from naïve animals were purified on a Histopaque 1.077 (Sigma) gradient and enriched for T cells by passing through a nylon wool column. T cells were cultured alone or in the presence of an equal number of purified MDSC and pulsed with BrdU for the last 24 h. Each culture condition was performed in triplicate. Cells were collected; sequentially stained with anti-CD3 and anti-BrdU mAbs; and analyzed by flow cytometry with initial gating on the CD3$^+$ population. Contact between T cells and MDSC was prevented by the use of 0.4 μM transwell inserts (Corning Inc., Lowell, MA). The culture medium from the add-back experiments was collected and used in subsequent assays for the determination of interferon (IFN)-γ and NO levels.

The incorporation of ^{3}H-thymidine was also used as a measure of proliferation in stimulated TIL cultures. TIL (1×10^6/ml RPMI medium) were seeded in 96-well plates in the presence of titrated doses of protein inhibitors (10–100 μM); neutralizing anti-TGF-β mAbs (0.5–5.0 μg/ml, R&D Systems, Inc., Minneapolis, MN); vehicle or non-specific murine IgG. Inhibitors used were: N-hydroxy-noroL-arginine (nor-NOHA, Calbiochem/EMD Chemicals, Inc., Gibbstown, NJ); methylthiohydantoin-DL-tryptophan (MTH-trp, Research Organics, Cleveland, OH); N-monomethyl-L-arginine (L-NMMA, Calbiochem) and indomethacin (Sigma). TIL were cultured for 3 days; pulsed with 1 μCi of ^{3}H-thymidine for the last 15 h. ^{3}H-thymidine incorporation was analyzed using a 96-well plate harvester and a beta-plate reader (Packard, Meridien, CT). Data are expressed as mean cpm of triplicate experimental cultures, ± SEM.