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GM-CSF Production by Glioblastoma Cells Has a Functional Role in Eosinophil Survival, Activation, and Growth Factor Production for Enhanced Tumor Cell Proliferation

Colleen S. Curran,* Michael D. Evans,† and Paul J. Bertics*,‡

Medicinal interventions of limited efficacy are currently available for the treatment of glioblastoma multiforme (GBM), the most common and lethal primary brain tumor in adults. The eosinophil is a pivotal immune cell in the pathobiology of atopic disease that is also found to accumulate in certain brain tumors. Inverse associations between atopy and GBM risk suggest that the eosinophil may play a functional role in certain tumor immune responses. To assess the potential interactions between eosinophils and GBM, we cultured human primary blood eosinophils with two separate human GBM-derived cell lines (A172, U87-MG) or conditioned media generated in the presence or absence of TNF-α. Results demonstrated differential eosinophil adhesion and increased survival in response to coculture with GBM cell lines. Eosinophil responses to GBM cell line-conditioned media included increased survival, activation, CD11b expression, and S100A9 release. Addition of GM-CSF neutralizing Abs to GBM cell cultures or conditioned media reduced eosinophil adhesion, survival, and activation, linking tumor cell-derived GM-CSF to the functions of eosinophils in the tumor microenvironment. Dexamethasone, which has been reported to inhibit eosinophil recruitment and shrink GBM lesions on contrast-enhanced scans, reduced the production of tumor cell-derived GM-CSF. Furthermore, culture of GBM cells in eosinophil-conditioned media increased tumor cell viability, and generation of eosinophil-conditioned media in the presence of GM-CSF enhanced the effect. These data support the idea of a paracrine loop between GM-CSF–producing tumors and eosinophil-derived growth factors in tumor promotion/progression. The Journal of Immunology, 2011, 187: 1254–1263.

Eosinophils are terminally differentiated granulocytic innate immune cells, originally characterized by Paul Ehrlich in 1879 (1). Of note, the main component of eosinophil primary granules was first described in Charcot and Robin’s 1853 postmortem examination of a leukemia patient (2). Subsequent findings in asthmatic sputum by Leyden in 1872 resulted in the present-day nomenclature of Charcot-Leyden crystals (3) and the first insight into a potential link between eosinophils and the inflammatory responses associated with cancer and asthma.

Blood and tissue eosinophils are now extensively reported in many types of human cancers (4, 5) and are well established contributors to the pathology of asthma and allergy (6). Cancer may form in response to chronic inflammation or promote inflammation through the activation of oncogenes (7). Although the role of eosinophils in these processes is not yet clear, eosinophil recruitment to the tumor microenvironment has been indicated to occur in response to necrosis, tumor-secreted IL-5, IgE Abs, and therapeutic treatment with IL-2, IL-4, or GM-CSF (5, 8–10). In asthma, the immune response has been characterized by early-phase IgE-mediated activation of mast cells, the production of proinflammatory cytokines (e.g., IL-2, IL-4, IL-5, GM-CSF), and the late-phase recruitment of Th2 cells and eosinophils (11). Evidence of an inverse relationship between atopic disease and the development of a particular cancer, glioblastoma (12–15), suggests that the eosinophil or eosinophilic mediators, or both, may play a pivotal role in an anticancer response.

Glioblastoma multiforme (GBM) is the most common and lethal primary brain tumor in adults despite the available cancer treatment of surgical resection, radiotherapy, and chemotherapy (16). Glioblastoma tumor cells are reportedly able to evade surgical, radiotherapeutic, chemotherapeutic, and immunotherapeutic interventions by respectively infiltrating into the surrounding brain tissue, downregulating tumor-suppressor proteins, upregulating DNA repair enzymes, and producing immunosuppressive cytokines (17). Notably, enhanced glioblastoma patient survival has been correlated with tissue eosinophilia in clinical trials involving postoperative treatments with IL-2 (18, 19). In animal models, transplanted glioblastoma tumor cells that express a high level of IL-2, IL-4, or GM-CSF displayed enhanced survival, reduced tumor growth, and significant eosinophil infiltrate compared with controls (20–22).

Eosinophil recruitment has also been indicated to occur in response to developing subdural hematomas (23), necrotic tissue (24), and radiotherapy (25), conditions known to exist in human primary GBM (16, 26). In patients with allergy and asthma, eosinophil recruitment involves cytokines (IL-3, IL-5, GM-CSF) priming in the peripheral blood that sensitizes eosinophilic adhesion molecules (CD11b/CD18, CD49d/CD29) to more effectively interact with adhesion ligands (ICAM-1, VCAM-1) on the
inflamed endothelia (27). Whether similar interactions occur in eosinophil recruitment to certain tumors is unclear. Understanding the distinct tumor microenvironments that encourage eosinophil infiltration may lead to more effective treatment parameters. Therefore, the aim of this study was to examine potential paracrine interactions between human primary eosinophils and glioblastoma cells, with a particular focus on the cytokine GM-CSF.

Materials and Methods

Isolation of peripheral blood eosinophils

Peripheral blood was obtained from human allergic patients under informed consent. The study was approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Eosinophils were purified from heparinized blood that was diluted with HBSS (Mediatech, Manassas, VA) without Ca2+ and centrifuged for 20 min at 700 × g over 1.090 g/ml Percoll. A granulocyte fraction was obtained after removal of the plasma, mononuclear cell band, and Percoll. Granulocytes were then subjected to RBC lysis via hypotonic shock, washed with 4˚C HBSS containing 1% BSA (Sigma Chemical) and treated (1 g Ab/1 ml 3% PBS) with anti-CD16, anti-CD14, and anti-CD3 (Miltenyi Biotechnology, Auburn, CA) before negative selection with an AutoMACS separator (Miltenyi Biotechnology). The recovered mixture (>97% purity, >98% viability) was evaluated by Giemsa’s-diff-Quik stain (Baxter Scientific Products, McGav Park, IL) and trypan blue exclusion, respectively.

Cell lines, cell culture, and reagents

The A172 and U87-MG glioblastoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). The H358 non-small cell lung cancer (NSCLC) and DU145 prostate carcinoma cell lines were kindly provided by Drs. Paul Harari and Wade Bushman (University of Wisconsin, Madison, WI), respectively. Eosinophils and cell lines were cultured in DMEM (Mediatech) supplemented with 10% cosmic calf serum (CCS; HyClone, Logan, UT), 100 U/ml penicillin/streptomycin (Mediatech) at 37°C, 5% CO2. Cells were treated with GM-CSF (R&D Systems, Minneapolis, MN), TNF-α (R&D Systems), or dexamethasone (Sigma Chemical, St. Louis, MO).

ELISA

Anti–GM-CSF mAbs (clone 6804; 1:1000; R&D Systems) in 0.1 M sodium carbonate buffer, pH 9.6, were coated onto 96-well enzyme immunoassay/radioimmunoassay plates (Costar, Corning, NY). Blocking buffer containing 1% BSA (Sigma Chemical) and 0.5% Tween 20 (Fischer Scientific, Pittsburgh, PA) in PBS was added to wells for 2 h. Serial dilutions of GM-CSF standard (215-GM; R&D Systems) and cell-free supernatants were aliquoted and incubated at 4°C overnight. GM-CSF was detected with biotinylated anti-GM-CSF Ab (clone 3209; 1:1000; R&D Systems) and subsequent exposure to streptavidin HRP-40 (Fitzgerald Industries International, Concord, MA). A colorimetric HRP substrate tetramethylbenzidine (Biofx Laboratories, Owings Mills, MD) was used to evaluate captured HRP activity, and the enzymatic reaction was stopped with 0.18 M sulfuric acid. OD was determined on an ELx800 Universal Microplate Reader (BioTek Instruments, Winooski, VT). Absorbance was quantified at 450 nM, using 600 nM as a reference wavelength. GM-CSF concentrations were calculated by interpolation from a standard curve, and all determinations were performed in triplicate.

Flow cytometric analysis of cell surface molecules

Tumor cell lines or eosinophils were suspended in 100 µl DMEM containing 1% CCS and treated (1 µg Ab/1 × 106 cells) with unconjugated mouse anti-human ICAM-1 Abs (clone BBIG1-11(C181); R&D systems), FITC-conjugated CD69 Abs (clone FN50; BD Biosciences Pharmingen, San Jose, CA), PE-conjugated CD11b Abs (clone ICRF44; BD Biosciences Pharmingen), or isotype control, and incubated for 30 min at 4°C. Cells were washed with 1 ml 1% CCS DMEM. For analysis of ICAM-1, which involved unconjugated primary Abs, PE goat anti-mouse Abs (Invitrogen, Eugene, OR) were used as a secondary and incubated for 30 min at 4°C in the dark. Cells were suspended in PBS, treated with propidium iodide (3 µg/ml) to exclude dead cells, and analyzed at 10,000 events for the fluorescence cytometer (Becton-Dickinson, Bedford, MA) at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Core Facility. Data were analyzed with FlowJo data analysis software (TreeStar, Ashland, OR).

Assay of eosinophil peroxidase activity

Eosinophil adherence was determined by measuring the eosinophil peroxidase (EPO) activity of adherent cells using a modification of the methods previously described (28–30). In triplicate, 100 µl media (10% CCS DMEM) or glioblastoma cells (5 × 105/ml) were added to 96-well tissue-culture plates (Sarstedt, Newton, NC). After 24-h incubation, eosinophils were incubated at 37°C for 30 min. The plate was washed twice with 200 µl HBSS, and 100 µl HBSS was aliquoted to each well. An eosinophil standard was plated in triplicate (1 × 105 eosinophils/ml) and serially diluted in HBSS. Reaction buffer (100 µl) containing 0.1% Triton X-100 (Sigma Chemical), 50 mM Tris (pH 8), 1 mM H2O2, and 1 mM O-phenylenediamine dihydrochloride (Sigma Chemical) was added to each well for an additional 30 min. The reaction was stopped with 4 M H2SO4 (50 µl), and OD was measured at 490 nm. The absorbance levels of EPO activity were used as an indirect measurement of eosinophil adherence by subtracting background values and interpolating the relative number of cells adhered from the standard curve.

Immunofluorescence

Glioblastoma cells (1 × 105/ml) were labeled with 1 µM Cell Trace CFSE (C34554; Invitrogen-Molecular Probes, Eugene, OR) per manufacturer’s instructions, and plated on coverslips (5 × 105/ml) and incubated at 37°C. After 24-h incubation, 10 ng/ml TNF-α or buffer control was added to media or glioblastoma cells for an additional 24 h at 37°C. At the 48-h time point, 100 pg/ml GM-CSF, 10 µg/ml GM-CSF neutralizing Abs (clone BVD2-23B6; Invitrogen), or isotype control Abs were added for 1 h. Eosinophils were suspended in HBSS (Mediatech) and added to the plate in 10-µl aliquots for a final concentration of 1 × 105 eosinophils/ml. Additional eosinophils were saved on ice as a standard. Dyes from the 96-well plate containing the samples were incubated at 37°C for 30 min. The plate was washed twice with 200 µl HBSS, and 100 µl HBSS was aliquoted to each well. An eosinophil standard was plated in triplicate (1 × 105 eosinophils/ml) and serially diluted in HBSS. Reaction buffer (100 µl) containing 0.1% Triton X-100 (Sigma Chemical), 50 mM Tris (pH 8), 1 mM H2O2, and 1 mM O-phenylenediamine dihydrochloride (Sigma Chemical) was added to each well for an additional 30 min. The reaction was stopped with 4 M H2SO4 (50 µl), and OD was measured at 490 nm. The absorbance levels of EPO activity were used as an indirect measurement of eosinophil adherence by subtracting background values and interpolating the relative number of cells adhered from the standard curve.

Eosinophil viability assay

Human glioblastoma cells (5 × 105 cells/0.5 ml) were cultured above 150 µl 0.5% w/v LE analytical grade agarose (Promega, Madison, WI), 48-well plate (Sarstedt). At 24 h, 500 µl eosinophils (2 × 105 cells) was added to semisolid glioblastoma spheres for an additional 96 h. In some experiments, GBM cell line-conditioned media (5 × 105 cells/ml, 48 h), as described previously (31), was used instead of the glioblastoma spheres. Eosinophils were also cultured alone in the presence or absence of GM-CSF (100 pg/ml) as positive and negative controls, respectively. To block GM-CSF activity, we added neutralizing Abs (clone 3209; R&D Systems) or clone BVD2-23B6; Invitrogen) or isotype control (10 µg/ml) in some experiments. To deplete GM-CSF, we added neutralizing Abs (clone BVD2-23B6; Invitrogen) or conditioned media (40 min, 4°C) following incubation with anti-CD11b Abs (20 µg/ml; 30 min, 4°C) followed by 10 µg/ml anti-CD11b Abs (clone ICRF44; 150, 1 h; R&D Systems), washed three times for 10 min with TBST, and immunostained with DAPI nuclear acid stain (1:10,000; Invitrogen) and Alexa Fluor 594-labeled donkey anti-mouse Abs (1:2000; 1 h; Invitrogen). After washing three times for 10 min with TBST, coverslips were rinsed in dH2O and mounted onto slides with ProLong Gold antifade reagent (Invitrogen). Glioblastoma cells and eosinophils were respectively visualized at 488 and 594 nm using a Nikon confocal microscope. A total of five images of each coverslip were randomly taken. Eosinophils identified by red CD11b staining and blue DAPI staining from four different donors were counted and averaged.

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Glioblastoma-induced Eosinophil Responses

Comprehensive Cancer Center Flow Cytometry Core Facility. Data were analyzed with FlowJo data analysis software (TreeStar) as shown in a representative example (Supplemental Fig. 2).

Immunoblotting

For an assessment of receptor for advanced glycation end products (RAGE) expression, cells were solubilized with lysis buffer (1% NaDodSO4 [SDS]), 10 mM DTT, 0.5 mM Na3Vo4, 1 mM EDTA, 10% glycerol, 10 mM Tris, pH 8.0), sonicated, boiled (5 min), and 50 μg protein, as determined by Micro-BCA protein assay reagents (Thermo Scientific Pierce, Rockford, IL). All samples were loaded onto a 10% SDS-PAGE gel. For analysis of S100A9, human primary blood eosinophils (5 × 10⁷/ml) were cultured in 96-well tissue-culture plates (Starstedt) ± 100 pg/ml GM-CSF, 10 ng/ml TNF-α, or GBM cell line-conditioned media generated in the presence of absence of 10 ng/ml TNF-α. After 24 h, total eosinophil conditioned medium from a total of 5 × 10⁷ cells was isolated via centrifugation (400 × g, 5 min). Concentrated (10X) lysis buffer was added to cell-free conditioned media (1:10 dilution) and boiled (5 min). Total conditioned media were loaded onto a 15% SDS-PAGE gel. Proteins were transferred onto 0.45-μm Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) and incubated with Abs raised against human RAGE (MAB1145, 1:500; R&D Systems) or S100A9 (Calgranulin B [C-19]: sc-8114, 1:200; Santa Cruz Biotechnology). The immunoblots were washed and subsequently incubated with HRP-conjugated secondary Abs. Bound secondary Ab was visualized using the incubation of the membrane with Super Signal West chemiluminescent HRP substrate (Thermo Scientific Pierce) and an Epicam II darkroom UV equipped with a 12-bit cooled charge-coupled device camera (UVPl, LLL, Upland, CA). Luminescence was quantified and evaluated using ImageJ software (National Institutes of Health).

MTS assay

Metabolism of 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used as an index of cell viability. Glioblastoma cell lines (2 × 10⁵ cells/ml) were cultured in complete media in 96-well tissue-culture plates (Starstedt) for 4 h to allow for adherence at 37°C. Media were replaced with serum-free media for 24 h and subsequently replaced with 0.1% CCS DMEM ± 100 pg/ml GM-CSF, 10% CCS DMEM (live control), 10 mM sodium azide (NaN3) in 0.1% CCS DMEM (dead control), or eosinophil-conditioned media obtained from 2-d cultures of eosinophils (2 × 10⁷/ml) in 0.1% CCS DMEM (±100 pg/ml GM-CSF). Glioblastoma cells were incubated with specified controls or eosinophil-conditioned media for 48 h at 37°C. Each well was aspirated and replaced with 100 μl/ml PBS plus 20 μl/ml of the CellTiter 96 Non-Radioactive Cell Proliferation Assay compound in MTS inner salt, 2 mg/ml; G1111; Promega, Madison, WI) and an electron coupling reagent phenazine methosulfate (0.92 mg/ml; P9625; Sigma Chemical). After 1-h incubation at 37°C, the reduction of MTS to formazan in metabolically active cells was measured at an OD of 490 nm. Statistical analysis

Analyses in all experiments were assessed among conditions using mixed-effects ANOVA models with a fixed-effect covariate per condition and a random-effect covariate to account for within-patient correlation of measurements. A two-sided p value <0.05 was regarded as statistically significant. The SEM indicates interassay variability.

Results

Characterization of glioblastoma cell lines

In allergy and asthma, the cytokine GM-CSF has been indicated to induce the sensitization of eosinophil adhesion molecules (CD11b/CD18, CD49d/CD29) to more effectively interact with their ligands (27). These ligands include ICAM-1, VCAM-1, and possibly RAGE (27, 33). To begin to understand eosinophil recruitment mechanisms in response to tumor cells, we examined the human A172 and U87-MG GBM cells for their potential production of GM-CSF and expression of adhesion molecules (ICAM-1, VCAM-1, RAGE). In Fig. 1A, basal levels of GM-CSF production were not detectable with the A172 cells, whereas the U87-MG cells produced >100 pg/ml GM-CSF during the 48-h time span. Addition of TNF-α to the cells enhanced the production of GM-CSF by either cell line, most significantly for the U87-MG cells. Likewise, in Fig. 1B, basal levels of adhesion molecules were not detectable in the A172 cells, whereas ICAM-1 was significantly expressed by the U87-MG cells. Addition of TNF-α to the cells induced ICAM-1 and VCAM-1 expression in the A172 cells but enhanced only ICAM-1 expression in the U87-MG cells. In examining RAGE expression, the A172 and U87-MG cell lines were compared with known positive (DU-145 prostate carcinoma cell line) (34) and negative (H358 NSCLC cell line) (35) controls. Fig. 1C indicates that both the A172 and U87-MG cell lines express RAGE, and that the U87-MG cells display 5-fold greater levels of this molecule when compared with the A172 cells. These data suggest that in the absence of stimuli, such as TNF-α, the U87-MG cell line may be able to induce stronger eosinophil adhesion responses in culture.

Eosinophil adhesion as measured by EPO activity in response to glioblastoma cocultures

To assess whether the U87-MG cell line induces preferential eosinophil adhesion compared with the A172 cell line, we performed EPO assays as an indirect measurement of adherence. Fig. 2A displays data revealing that eosinophil adherence to both tumor cell

**FIGURE 1.** Characterization of human A172 and U87-MG glioblastoma cell lines. A, GM-CSF ELISA of supernatants collected from tumor cell lines (5 × 10⁵ cells/ml) plated 24 h and treated with buffer control or 10 ng/ml TNF-α for an additional 24 h. The mean concentration ± SEM is displayed, n = 3. *p < 0.0001 versus control. B, Flow cytometry analysis of VCAM-1 and ICAM-1 geometric mean fluorescence intensity (GMFI) from tumor cell lines (5 × 10⁵ cells/ml) plated 24 h and treated with buffer control or 10 ng/ml TNF-α for an additional 24 h ± SEM, n = 3. *p < 0.0001 versus isotype control, †p < 0.0001 versus basal expression. C, A representative immunoblot and ImageJ assessment of GBM RAGE compared with positive (DU-145 prostate carcinoma) and negative (H358 NSCLC) controls. Data charted represent RAGE mean band densitometry relative to actin ± SEM, n = 5. *p ≤ 0.0006 versus H358, †p = 0.007 versus DU-145, ‡p = 0.002 versus A172.
lines was not significantly different from the media control. These data indicate that eosinophils do not preferentially adhere in response to coculture with the U87-MG cells compared with the A172 cells in a 30-min time frame. Pretreatment of the cell lines with TNF-α for 24 h enhanced eosinophil adhesion to the plate and to the A172 cells, but not to the U87-MG cells. Preincubation (1 h) with GM-CSF neutralizing Abs reduced adhesion, most significantly for the GM-CSF control and for the A172 cells stimulated with TNF-α. These results indicate that eosinophils are more adhesive in the presence of the TNF-α-treated A172 cells, which produce lower levels of the cytokine GM-CSF compared with U87-MG cells (Fig. 1A).

Immunofluorescence of eosinophil adherence in cocultures

To further assess the adhesive functions of eosinophils in cocultures with glioblastoma cell lines, we performed immunofluorescence assays. Similar to the results of the EPO assay, the TNF-α–treated A172 cell line appeared to induce eosinophil adherence after 10-min incubation with eosinophils. Addition of GM-CSF neutralizing Abs (clone BVD2-23B6) to TNF-α–treated A172 cell line cultures 1 h before the addition of eosinophils mitigated the adhesive response (Fig. 2B). Significant eosinophil adherence was not identified in control A172 cell line control.

FIGURE 2. Human blood eosinophil adherence as measured by EPO activity and immunofluorescence. A, Media controls (buffer, 100 pg/ml GM-CSF, 10 ng/ml TNF-α) or tumor cell lines stimulated (24 h) with buffer control or 10 ng/ml TNF-α were treated with an isotype control or GM-CSF neutralizing Ab (1 h) before adding eosinophils. An EPO assay was performed after 30-min incubation with eosinophils as described in Materials and Methods. Data represent the average relative number ± SEM of eosinophils adhered. n = 5. *p ≤ 0.0001 versus respective isotype control, †p ≤ 0.009 versus media control, ‡p ≤ 0.005 versus TNF media control, §p < 0.0001 versus A172 cell line control. B, Media controls (buffer, 100 pg/ml GM-CSF, 10 ng/ml TNF-α) or tumor cell line cultures stimulated (24 h) with buffer control or 10 ng/ml TNF-α were treated with an isotype control or GM-CSF neutralizing Ab (1 h) before adding eosinophils. After 10-min incubation with eosinophils, the cells were washed, fixed, and stained as described in Materials and Methods. Data represent the average number ± SEM of eosinophils adhered. n = 4. *p ≤ 0.0001 versus respective isotype control, †p ≤ 0.0006 versus media control, ‡p ≤ 0.004 versus TNF media control, §p < 0.0001 versus A172 cell line control.

FIGURE 3. Human blood eosinophil viability in response to coculture with A172 and U87-MG spheroids. Human blood eosinophils (2 × 10⁷/ml) were cultured above 0.5% w/v agarose, 48-well plate, ± 100 pg/ml GM-CSF or glioblastoma cells (5 × 10⁴/ml) for 4 d. A, Cells were positively selected for CD11b expression and negatively selected for propidium iodide stain. The percentage average viability ± SEM is displayed. n = 4. *p < 0.0001 versus media control. B, Cells cultures were resuspended in 50 µl media and diluted 1:2 in trypan blue. Cells were identified as viable via trypan blue exclusion. The percentage average viability ± SEM is displayed. n = 3. *p < 0.0001 versus media control. C, Supernatants from 4-d cultures were assessed for the presence of GM-CSF via ELISA (as detailed in Materials and Methods). The mean concentration ± SEM is displayed. n = 3. *p < 0.0001 versus all other samples.
cultures (Fig. 2B) or U87-MG cultures generated in the presence or absence of TNF-α (data not shown).

**Eosinophil viability in response to glioblastoma spheroid coculture**

Tumor cell lines suspended above agar are known to form spheroids, involving a diverse population of quiescent, hypoxic, and necrotic cells similar to human tumors and equally resistant to radiation and experimental drugs (36, 37). Spheroids in culture with immune cells have previously been used to model the tumor microenvironment and examine potential immunotherapeutic treatments (38). A previous study has also identified enhanced eosinophil viability in cocultures of human biologically active GBM explants and autologous peripheral blood leukocytes (39). To examine whether growing tumor spheroids affect eosinophil viability, we cocultured eosinophils with GBM spheroids for 4 d. Tumor cell lines were evaluated for CD11b expression via flow cytometry analysis and determined to be negative for this cell adhesion molecule (data not shown). The expression of CD11b has been previously characterized on eosinophils (40) and was therefore used as a positive selection marker in identifying eosinophils in cocultures. As a positive indicator of viability, GM-CSF was added to eosinophil monocultures similar to our previous study (41). Viability was determined by the absence of propidium iodide stain, revealing increased eosinophil viability from four different patients in the presence of GM-CSF or GBM spheroids (Fig. 3A). These data were confirmed with three additional patients via analysis of live cells using a trypan blue exclusion assay (Fig. 3B). Concomitant examination of 4-d culture supernatants by ELISA revealed the presence of GM-CSF. Notably, as shown in Fig. 3C, we observed that the diminished levels of GM-CSF found in the positive control could still maintain eosinophil viability. Significant production of GM-CSF in U87-MG cell cultures did not further alter eosinophil viability compared with

**FIGURE 4.** Human blood eosinophil viability in response to GBM cell line-conditioned media. Human blood eosinophils (2 × 10^6/ml) were cultured above 0.5% w/v agarose, 48-well plate, ±100 pg/ml GM-CSF or GBM cell line-conditioned media for 4 d. Cells were gated (as shown in Supplemental Fig. 2) and negatively selected for propidium iodide stain. A, The percentage average viability in response to media controls or conditioned media is displayed. n = 5. *p ≤ 0.001 versus media control. B, The percentage average viability in response to coculture with anti–GM-CSF (clone BVD2-23B6) or isotype Abs is displayed. n = 4. *p ≤ 0.0001 versus media control. C, The percentage average viability in response to GBM cytokine depletion (clone BVD2-23B6) or isotype controls is displayed. n = 3. *p ≤ 0.0001 versus media control.

**FIGURE 5.** Human blood eosinophil CD69 expression in response to GBM cell line-conditioned media. Human blood eosinophils (2 × 10^6/ml) were cultured above 0.5% w/v agarose, 48-well plate, ±100 pg/ml GM-CSF or GBM cell line-conditioned media generated in the presence or absence of 10 ng/ml TNF-α. In some wells, 10 μg/ml anti–GM-CSF (clone BVD2-23B6) or isotype Abs were added. Cultures were maintained for 3 h. Cells were gated (as shown in Supplemental Fig. 4), negatively selected for propidium iodide stain, and positively selected for CD69 expression. A, The percentage average CD69 expression ± SEM in response to media controls or conditioned media is displayed. n = 5. *p < 0.0001 versus media, †p < 0.0001 versus TNF-α, ‡p < 0.0001 versus A172, †p < 0.0001 versus U87-MG. B, The percentage average CD69 expression ± SEM in response to coculture with anti–GM-CSF (clone BVD2-23B6) or isotype Abs is displayed. n = 3. *p < 0.0001. C, The percentage average CD69 expression ± SEM in response to GBM cytokine depletion (clone BVD2-23B6) or isotype controls is displayed. n = 4. *p < 0.0001.
the positive control or the observations associated with the A172 cell cultures. These results suggest that the very low levels of GM-CSF in the A172 cell cultures are sufficient for maximum viability and/or that another factor(s) may contribute to eosinophil viability in the A172 cell cultures.

Eosinophil viability in response to GBM cell line-conditioned media

Soluble factors derived from tumor cells have been found to affect the activation of human monocytic cells (31). To test the idea that soluble factors produced by tumor cells induced eosinophil survival as suggested by the data in Fig. 3, eosinophils were cultured in GBM cell line-conditioned media for 4 d and analyzed by flow cytometry. As shown in Fig. 4A, GBM cell line-conditioned media are also able to induce the survival of eosinophils from five different patients, similar to the GM-CSF positive control, comparable with spheroid coculture (Fig. 3A, 3B), and despite differential production levels of GM-CSF between tumor cell lines (Fig. 3C).

Effect of GM-CSF neutralization on GBM cell line-conditioned media-induced eosinophil viability

To assess whether the soluble factor involved in GBM cell line-conditioned media-induced viability is GM-CSF, we added neutralizing Abs from two separate vendors to 4-d cultures. As shown in Fig. 4B, GBM cell line-conditioned media-induced eosinophil viability from four different patients is reduced in the presence of GM-CSF neutralizing (clone BVD2-23B6) Abs but not in the presence of the isotype controls. Additional viability analyses of human blood eosinophils from three different patients after GM-CSF neutralization using an Ab from an alternative source (clone 3209) confirmed these results (Supplemental Fig. 3). These combined data suggest that low levels of GM-CSF, produced by tumor cell lines or eosinophils in response to the conditioned media, are able to enhance eosinophil survival.

Effect of GM-CSF depletion on GBM cell line-conditioned media-induced eosinophil viability

Because eosinophils have been suggested to produce GM-CSF in response to various stimuli (42, 43), GM-CSF cytokine depletion of tumor cell-conditioned media was performed before culturing eosinophils. As shown in Fig. 4C, GBM cell line-conditioned media-induced eosinophil viability from four different patients is reduced after GM-CSF cytokine depletion but not in response to the isotype controls. These data suggest that GM-CSF–mediated viability responses are a function of tumor-derived GM-CSF.

FIGURE 6. Human blood eosinophil CD11b expression in response to GBM cell line-conditioned media. Human blood eosinophils (2 × 10^5/ml) were cultured above 0.5% w/v agarose, 48-well plate, ±100 pg/ml GM-CSF, 10 ng/ml TNF-α, or GBM cell line-conditioned media generated in the presence or absence of 10 ng/ml TNF-α. Cultures were maintained for 24 h. A, Cells were negatively selected for propidium iodide stain and positively selected for CD11b expression. B, The percentage average CD11b expression ± SEM in response to media controls or conditioned media is displayed. n = 3. *p = 0.01 versus control, †p < 0.03 versus TNF-α.
Eosinophil CD69 expression in response to GBM cell line-conditioned media

A strong inducer of eosinophil activation is GM-CSF, as exhibited by CD69 expression (44). To examine whether GBM cell line-conditioned media also induce CD69 expression, we cultured eosinophils with GBM cell line-conditioned media for 3 h before analysis via flow cytometry. Because previous research has indicated that TNF-α induces GM-CSF production by GBM cells (45), 10 ng/ml TNF-α was added to selected GBM cultures 24 h before harvesting the GBM cell line-conditioned media. As shown in Fig. 5A, significant eosinophil CD69 expression from six different patients occurred in response to U87-MG, but not A172 cell-conditioned media, compared with media alone and the GM-CSF media control. Addition of TNF-α during the generation of GBM cell line-conditioned media induced CD69 expression in A172 cultures and enhanced the expression in U87-MG cultures compared with TNF-α and respective GBM cell line-conditioned media alone. The absence of CD69 expression in the presence of A172 cell-conditioned media may be a response to the lower basal levels of GM-CSF produced by these cells compared with the U87-MG (Fig. 1A).

Effect of GM-CSF neutralization on GBM cell line-conditioned media-induced eosinophil CD69 expression

To determine whether tumor-derived GM-CSF induces eosinophil CD69 expression, neutralizing Abs to GM-CSF were added to eosinophils cultured in GBM cell line-conditioned media and compared with a GM-CSF positive control. As shown in Fig. 5B, eosinophil CD69 expression from four different patients is reduced in the presence of GM-CSF neutralizing Abs with significant reductions found in TNF-α–generated conditioned media, the U87-MG-conditioned media alone, and the GM-CSF control.

Effect of GM-CSF depletion on GBM cell line-conditioned media-induced eosinophil CD69 expression

Because eosinophils have been indicated to produce GM-CSF in response to various stimuli (42, 43), GM-CSF cytokine depletion of tumor cell-conditioned media was performed before culturing eosinophils. As shown in Fig. 5C, GBM cell line-conditioned media-induced eosinophil CD69 expression from three different patients is reduced after GM-CSF cytokine depletion, but not in response to the isotype controls, similar to Fig. 5B. These data suggest that GM-CSF–induced CD69 expression is a function of tumor cell-derived GM-CSF and not eosinophil autocrine activity.

GBM cell line-conditioned media-induced eosinophil CD11b expression

The eosinophil adhesion molecule CD11b is known to be responsive to GM-CSF (27). To test whether GBM cell line-conditioned media affects eosinophil CD11b expression, we cultured eosinophils with GBM cell line-conditioned media for 24 h before analysis via flow cytometry. As shown in Fig. 6, significant eosinophil CD11b expression from three different patients occurred in response to U87-MG but not A172 cell-conditioned media. Addition of TNF-α during the generation of GBM cell line-conditioned media induced significant CD11b expression in A172 cell cultures and maintained U87-MG CD11b expression compared with media alone, GM-CSF, and TNF-α treatments.

GBM cell line-conditioned media-induced eosinophil S100A9 release

Induction of CD11b is reportedly dependent on the presence of a cytoplasmic calcium-binding protein, S100A9 (46). Release of S100A9 in the tumor microenvironment may be tumorigenic since a concentration of 5 × 106/ml) were cultured with media ± 100 pg/ml GM-CSF (G), 10 ng/ml TNF-α (T), or GBM cell line-conditioned media generated in the presence or absence of 10 ng/ml TNF-α for 24 h. Total eosinophil cell-free conditioned media or media controls were loaded onto a 15% gel. A representative immunoblot and ImageJ assessment of S100A9 are displayed. As shown in Fig. 7, S100A9 was identified in cocultures involving media controls and GBM cell line-conditioned media generated in the presence or absence of TNF-α from three different patients.

Effect of dexamethasone on GM-CSF production by tumor cell lines

Eosinophil recruitment to the lung is reduced by dexamethasone (49), a common corticosteroid administered to GBM patients with peritumoral edema (50). Dexamethasone is also indicated to inhibit the release of GM-CSF from human primary T cells (51), suggesting that similar responses may occur in tumor cell lines known to produce GM-CSF. To test whether dexamethasone affects GBM cell line GM-CSF production, we plated 5 × 105 cells/ml for 24 h before the addition of vehicle control, 10 ng/ml TNF-α, and increasing concentrations of dexamethasone, followed by an additional 24-h incubation. As shown in Fig. 8, TNF-α–induced, but not basal, GM-CSF production by the GBM tumor cell lines is significantly reduced by dexamethasone. Tumor cell line viability was not inhibited by dexamethasone or TNF-α treatments (Supplemental Fig. 5).

Tumor cell line growth in response to eosinophil-conditioned media

Eosinophil-conditioned media generated in the presence or absence of GM-CSF has been shown to enhance endothelial cell proliferation (52). To assess whether soluble factors produced by eosinophils affect glioblastoma cell growth, we cultured cell lines with eosinophil-conditioned media and subjected them to an MTS assay. As shown in Fig. 9, soluble factors from eosinophils significantly induced glioblastoma cell growth compared with controls. These conditions resulted in 1.7- and 1.3-fold increases in respective A172 and U87-MG viability over the media control. Generation of eosinophil-conditioned media in the presence of 100 pg/ml GM-CSF enhanced the effect with 2.1- (A172) and 1.6-fold (U87-MG) increases in viability over the GM-CSF media control.

Discussion

The reported inverse correlation between atopic disease and glioblastoma risk (12–15) suggests that an immune cell pivotal to glioblastoma risk (12–15) suggests that an immune cell pivotal to glioblastoma risk (12–15) suggests that an immune cell pivotal to the regulation of tumor cell proliferation is eosinophils. GM-CSF production by GBM cells (42, 43), GM-CSF cytokine depletion of tumor cell-conditioned media was performed before culturing eosinophils. As shown in Fig. 5C, GBM cell line-conditioned media-induced eosinophil CD69 expression from three different patients is reduced after GM-CSF cytokine depletion, but not in response to the isotype controls, similar to Fig. 5B. These data suggest that GM-CSF–induced CD69 expression is a function of tumor cell-derived GM-CSF and not eosinophil autocrine activity.
A172 and U87-MG cell line proliferation in response to human blood eosinophil-conditioned media. Human blood eosinophil-conditioned media was generated by culturing \(2 \times 10^6\) cells/ml in 0.1% CCS DMEM ± 100 pg/ml GM-CSF, 24-well plate, 48 h. Serum-starved tumor cell lines (\(2 \times 10^5\)/ml) were cultured in 100 \(\mu\)l eosinophil-conditioned media, media controls, 10% DMEM (live control), 10 mM sodium azide (dead control), 96-well plate, 48 h. Culture supernatant fluids were examined in triplicate for GM-CSF via ELISA. The mean concentration ± SEM is displayed. \(n = 5\). *\(p \leq 0.007\) versus respective media controls, 10% DMEM (live control), 10 mM sodium azide (dead control), 96-well plate, 48 h. Culture supernatant fluids were examined in triplicate for GM-CSF via ELISA. The mean concentration ± SEM is displayed. \(n = 5\). *\(p \leq 0.007\) versus respective media controls. The allergenic response, such as the eosinophil (6), may also function in an anticancer response. Because cytokines such as GM-CSF and adhesion molecules (CD11b/CD18, CD49d/CD29) function in the recruitment of eosinophils in atopic disease (27), similar mechanisms may be essential to the migration of eosinophils into tumor tissue. Examination of glioblastoma cell lines indicated that the U87-MG but not the A172 cell line expressed significant basal levels of GM-CSF and ICAM-1, and higher levels of RAGE (Fig. 1). These data suggest that CD11b-expressing eosinophils (Fig. 6) may be more inclined to interact with the U87-MG compared with the A172 cell line. Assessment of eosinophil viability (Fig. 3A, 3B) revealed increased eosinophil survival in cocultures compared with controls, irrespective of the glioblastoma cell type.

To determine whether the enhanced eosinophil viability in glioblastoma cocultures was a product of interactions between the cell types or soluble factors within the media, we generated GBM glioblastoma cocultures was a product of interactions between the cell types or soluble factors within the media, we generated GBM glioblastoma cocultures. Assess-

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previously assessed human brain tissue biopsy specimens (58). Dexamethasone has also been reported to reduce eosinophil recruitment (49), induce eosinophil apoptosis (59), and inhibit eosinophil TNF-α–induced GM-CSF production (42). Focal expression of TNF-α has been identified in infiltrating leukocytes in GBM tumors (60). Dexamethasone therapy (16 mg/day) in some GBM cases reduced the imaging of lesions on contrast-enhanced scans (61, 62), possibly by inhibiting immune cell recruitment via reduced capillary permeability at the brain–tumor barrier (63), inducing eosinophil apoptosis (59), and/or reducing localized GM-CSF production (Fig. 8) (42). Thus, the efficacy of dexamethasone treatment may be a factor of the tumor microenvironment and whether the established immune response is tumorigenic or immunogenic.

In examining the eosinophil immune response, GBM cell lines were cultured with eosinophil-conditioned media and assessed for proliferation via MTS assay. As shown in Fig. 9, eosinophil-conditioned media, generated in the presence or absence of GM-CSF, enhanced glioblastoma cell growth compared with respective controls. These data are supported by examining A172 CFSE staining (Supplemental Fig. 6). After 48-h culture with eosinophil-conditioned media, generated in the presence or absence of GM-CSF, the A172 cells exhibited reduced CFSE expression compared with media controls, suggesting increased proliferation (p < 0.02). Eosinophils stimulated with GM-CSF are known to produce amphiregulin and TGF-α, ligands highly implicated in tumor promotion via a common receptor, epidermal growth factor receptor (EGFR) (64–66). In primary GBM, amplification of the EGFR gene and subsequent overexpression of EGFR protein is the most common genetic alteration (67). Release of S100A9 (Fig. 7) was identified in response to GBM cell line-conditioned media, and interestingly, this protein is also increased in response to radiotherapy in GBM studies (68). This work is consistent with the idea that S100A9 may interact with GBM-associated RAGE and downstream signaling (69). Eosinophils are known to produce GM-CSF in response to GM-CSF treatment (70), a paracrine loop may be a factor of the tumor microenvironment and whether the established immune response is tumorigenic or immunogenic.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

13. Tsallil, R., G. D. Majhail, and T. R. Leung. 2007. Dexamethasone has also been reported to reduce eosinophil apoptosis (59), and inhibit eosinophil TNF-α–induced GM-CSF production (42). Focal expression of TNF-α has been identified in infiltrating leukocytes in GBM tumors (60). Dexamethasone therapy (16 mg/day) in some GBM cases reduced the imaging of lesions on contrast-enhanced scans (61, 62), possibly by inhibiting immune cell recruitment via reduced capillary permeability at the brain–tumor barrier (63), inducing eosinophil apoptosis (59), and/or reducing localized GM-CSF production (Fig. 8) (42). Thus, the efficacy of dexamethasone treatment may be a factor of the tumor microenvironment and whether the established immune response is tumorigenic or immunogenic.

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In summary, we have shown that eosinophils are more viable and activated in the presence of GBM tumor cell lines or GBM cell line-conditioned media. These eosinophil responses are, in part, regulated by tumor cell-derived GM-CSF as indicated by neutralization and cytokine depletion experiments. Eosinophils, in the presence or absence of GM-CSF, produced growth factors essential to tumor cell viability, indicating a potential cooperative function of eosinophils in tumor promotion/progression. Thus, the inverse correlations reported between atopic disease and GBM risk cannot be attributed to the functional responses of eosinophils alone. The enhanced GBM production of GM-CSF and expression of adhesion ligands (ICAM-1, VCAM-1) in the presence of TNF-α suggest a synergy with microglial cells in the recruitment and activation of eosinophils. These findings offer insight into GBM and other GM-CSF secreting tumors [colon (71), prostate (72), and skin (73)] known to recruit eosinophils (5, 24, 74), and emphasize the need to understand the immunological networks within the tumor microenvironment in developing more effective immunotherapeutic protocols.

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Supplemental Figure Legends

**Supplemental Figure 1. Imaging GBM spheres and eosinophils.** Glioblastoma cells were labeled red with PKH26 (2 µM, 050M0734, PKH26 Red Fluorescent Cell Linker Mini Kit, Sigma Chemical Co.) per manufacure instructions. Labeled glioblastoma cells (5x10^3 cells/ 100 µl) were cultured above 50 µl 0.5% w/v LE analytical grade agarose (Promega), 96-well plate (Sarstedt). After a 24 hr incubation, 10 ng/ml TNF-α or buffer control was added to media or glioblastoma cells for an additional 24 hr. At the 48 hr time point, 100 pg/ml GM-CSF was added to control wells and eosinophils were stained green with Cell Trace™ CFSE (2 µM, C34554, Invitrogen-Molecular Probes, Eugene, OR) per manufacturer instructions. Labeled tumor cells and eosinophils were cultured 30 min (A) or 60 min (B) and visualized on an Olympus IX71/IX51 inverted microscope (Olympus America, Melville, NY). Representative images from one of three independent experiments involving 3 different donors are displayed.

**Supplemental Figure 2. Eosinophil viability gating.** Eosinophils (2x10^5/ml) were cultured above 0.5% w/v agarose, 48-well plate, +/- 100 pg/ml GM-CSF, GBM spheroids or GBM-conditioned media. In some wells, 10 µg/ml anti-GM-CSF or isotype control antibodies were added to the cell culture or used for cytokine depletion. Cultures were maintained for 4 days. Cells were selected and analyzed on a log scale based on size and PE-CD11b expression in co-cultures or size alone in media cultures on a FACScan flow cytometer (Becton–Dickinson) at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Core Facility. Data were analyzed with FlowJo data analysis software (TreeStar). Live cell gates from a total of 10,000 events were established on GM-CSF treated samples in the absence of propidium iodide stain. Gates were then...
copied to all other samples. A representative example of cell gates involving eosinophils cultured in media controls, conditioned media, +/- neutralizing antibodies or isotype control are displayed.

**Supplemental Figure 3. Eosinophil viability in response to GBM-conditioned media and Clone 3209.** Eosinophils (2x10^5/ml) were cultured above 0.5% w/v agarose, 48-well plate, +/- 100 pg/ml GM-CSF or GBM-conditioned media for 4 days. Cells were gated (as shown in Supplementary Figure 2) and negatively selected for propidium iodide stain. The percent average viability in response to co-culture with anti-GM-CSF (Clone 3209) or isotype antibodies is displayed, N=3, *p<0.0001 vs media control.

**Supplemental Figure 4. Eosinophil CD69 gating.** GM-conditioned media (generated in the presence or absence of 10 ng/ml TNF-alpha) or GM-CSF media controls were cytokine depleted or treated with neutralizing antibodies and isotype controls prior to the addition of eosinophils (2x10^5/ml). Cells were cultured above 0.5% w/v agarose, 48-well plate. After 3 hr, 10,000 events were assessed on a FACScan flow cytometer (Becton–Dickinson) at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Core Facility. Data were analyzed on a log scale with FlowJo data analysis software (TreeStar). A dense population of cells was isolated (A) and negatively selected for propidium iodide stain (B). CD69 positive gates were established on GM-CSF treated samples and copied to all other samples. Control samples involving media alone (C), 10 ng/ml TNF-alpha (D) were compared against GM-CSF (100 pg/ml) and tumor conditioned medias (E). Representative histograms involving are displayed
from one representative experiment (C-E), involving eosinophils cultured in media controls, conditioned media, +/- neutralizing antibodies or isotype control.

**Supplemental Figure 5. Tumor cell line proliferation via MTS assay in response to dexamethasone.** Glioblastoma cells (5x10^5/ml) were plated in triplicate and treated with increasing concentrations of dexamethasone (Dex) +/- 10 ng/ml TNF-α, 96-well plate, 24 hr. Culture supernatant fluids were removed for examination of GM-CSF production via ELISA (see Figure 8) and 100 µl of phosphate buffered saline (PBS, pH 7.4) was added to each well. Cell proliferation was assessed via MTS assay as described in Materials and Methods. Average absorbance values are displayed, +/- SEM, N>4, *p<0.02.

**Supplemental Figure 6. Tumor cell line proliferation in response eosinophil conditioned media via CFSE assay.** Eosinophil conditioned media or media controls were prepared for culture with tumor cells. Eosinophils (2x10^6/ml) cultured in 0.1% CCS DMEM were treated with vehicle control or 100 pg/ml GM-CSF, 24-well plate, 48 hr. Simultaneously, Cell Trace™ CFSE (1 µM, C34554, Invitrogen-Molecular Probes, Eugene, OR) labeled A172 cells (2x10^5/ml) were serum starved (48 hr, 24-well plate). For an additional 48 hr, tumor cells were cultured in 1 ml of eosinophil conditioned media, media controls, or 10% CCS DMEM. Tumor cells were harvested at time points (0, 24, and 48 hr), fixed in 1% paraformaldehyde and assessed for proliferation via reduced CFSE expression. A total of 10,000 events were assessed on a FACScan flow cytometer (Becton–Dickinson) at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Core Facility. Data were analyzed on a log scale with FlowJo.
data analysis software (TreeStar). Data from one representative experiment are displayed in A-D where a central population was selected (A). Cells plated in 10% serum served as a positive proliferation control (B). A172 cells cultured in 0.1% CCS are compared to tumor cells cultured in eosinophil conditioned media (C) or A172 cells cultured in 0.1% CCS plus GM-CSF are compared to tumor cells cultured in eosinophil conditioned media generated in the presence of GM-CSF (D). Each experiment was subsequently charted displaying the media control CFSE geometric mean fluorescence intensity (GMFI) compared to conditioned media from 5 different donors in the presence (F) or absence (E) of GM-CSF, †p < 0.02 versus the average media control, ‡p < 0.001 versus the average GM-CSF control.