Abstract

Interaction of RAGE (the receptor for advanced glycation endproducts) with its ligands can promote tumor progression, invasion, and angiogenesis. Although blocking RAGE signaling has been proposed as a potential anticancer strategy, functional contributions of RAGE expression in the tumor microenvironment (TME) have not been investigated in detail. Here, we evaluated the effect of genetic depletion of RAGE in TME on the growth of gliomas. In both invasive and noninvasive glioma models, animal survival was prolonged in RAGE knockout (Ager−/−) mice. However, the improvement in survival in Ager−/− mice was not due to changes in tumor growth rate but rather to a reduction in tumor-associated inflammation. Furthermore, RAGE ablation in the TME abrogated angiogenesis by downregulating the expression of proangiogenic factors, which prevented normal vessel formation, thereby generating a leaky vasculature. These alterations were most prominent in noninvasive gliomas, in which the expression of VEGF and proinflammatory cytokines were also lower in tumor-associated macrophages (TAM) in Ager−/− mice. Interestingly, reconstitution of Ager−/− TAM with wild-type microglia or macrophages normalized tumor vascularity. Our results establish that RAGE signaling in glioma-associated microglia and TAM drives angiogenesis, underscoring the complex role of RAGE and its ligands in gliomagenesis.

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Introduction

With the development of targeted therapies against gliomas, the contribution of tumor microenvironment (TME) to treatment response is becoming clearer. Glioma-associated stromal cells like astrocytes, endothelial cells, mesenchymal cells and infiltrating inflammatory cells play an important role in tumor...

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regulation of cytokines, chemokines, adhesion molecules, and pathways that regulate cell proliferation, survival, differentiation, migration, phagocytosis, and autophagy (4). Thus, it is not surprising that RAGE signaling has also been implicated in tumorigenesis.

By facilitating the maintenance of a chronic inflammatory state, upregulation of RAGE and its ligands has been linked to the development and progression of several neoplasms, including gastric cancer (5), colon cancer (6), pancreatic cancer (7), and prostate cancer (8). Furthermore, blockade of RAGE signaling has been used to inhibit tumor growth, invasion, and angiogenesis in a variety of cancers (9–12). One of the earliest reports to demonstrate the therapeutic efficacy of this approach was in gliomas in which blockade of RAGE interaction with HMGB1 was shown to inhibit tumor growth and invasion (13). Most studies that have evaluated RAGE signaling in tumors, however, have focused on the RAGE signaling in neoplastic cells, and the contribution of RAGE expression in the TME to tumorigenesis has not been investigated in detail.

The goal of this study was to evaluate the effect of genetic depletion of RAGE in TME on the growth of gliomas. Because gliomas are heterogeneous tumors with both invasive and noninvasive "bulk" phenotypes, two different syngeneic glioma models were used for these experiments. In both glioma models, animal survival was prolonged in Ager−/− mice. This improvement in animal survival, however, was not due to changes in tumor growth, but to a reduction in tumor-associated inflammation in Ager−/− mice. Furthermore, RAGE ablation in TME abrogated angiogenesis by downregulating the expression of proangiogenic factors that prevented normal vessel formation. These vascular alterations, however, were most prominent in noninvasive gliomas in which the expression of VEGF and proinflammatory cytokines was lower in Ager−/− TAMs. Interestingly, reconstitution of either RAGE null microglia or macrophages with corresponding WT cells normalized tumor angiogenesis. These findings support the role of TAM RAGE signaling in glioma angiogenesis.

Materials and Methods

Reagents and cell lines

Murine GL261 glioma cells were obtained from the laboratory of Dr. Karen Aboody (City of Hope Beckman Research Institute, Duarte, CA) in 2006 and stably transfected with firefly luciferase expression vector as described previously (14, 15). Positive clones (GL261-luc) were selected using zeocin (1 mg/mL) and G418. Luciferase-expressing KR158B cells (or K-Luc), an invasive glioma cell line that was derived from spontaneous gliomas in Tprp53/Nfj double-mutant mice in the laboratory of Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA), were a generous gift from Dr. John Sampson (Duke University, Durham, NC) in 2011 (16). Both GL261 and K-Luc cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin-G, 100 μg/mL streptomycin, and 0.01 mol/L Hepes. Cathepsin S assay was tested for Cathepsin S activity.

Primary bone marrow–derived monocytes (BMM) were harvested from WT and Ager−/− mice as described previously (19) and cultured in DMEM supplemented with 1% FBS, 100 U/mL penicillin-G, 100 μg/mL streptomycin, and 0.01 mol/L Hepes. Cathepsin S assay was performed according to the manufacturer’s instructions (AnaSpec Inc.; catalog# 72099). Briefly, 106 BMM from WT and Ager−/− mice were plated in 12-well plated for 24 hours. Cells were then treated with IFNγ (1 μg/mL), or conditioned medium (CM) from GL261or K-Luc cells. Cell lysates were collected at different time points and tested for Cathepsin S activity.

Tumor implantation and imaging

Mice were housed and handled in accordance with the guidelines and approval of City of Hope Institutional Animal Care and Use Committee under pathogen-free conditions. All mice were on C57BL/6j background. Cx3cr1G/R− knock-in mice that express EGFP under control of the endogenous Cx3cr1 locus were purchased from The Jackson Laboratory. Ager−/− mice, a generous gift from Dr. Yasuhiko Yamamoto (Kanazawa University, Kanazawa, Japan), were bred at our institution and PCR genotyped using tail DNA (17). Intracranial tumor implantation was performed stereotactically as described previously (18). Briefly, GL261 or K-Luc glioma cells were harvested by trypsinization, counted, and resuspended in culture medium. Female C57BL/6 mice (The Jackson Laboratory) weighing 15 to 25 g were anesthetized by i.p. administration of ketamine (132 mg/kg) and xylazine (8.8 mg/kg), and implanted with 106 tumor cells using a stereotactic head frame at a depth of 3 mm through a bur hole placed 2-mm lateral and 0.5-mm anterior to the bregma. Tumor growth was assessed by the Xenogen In Vivo Imaging System (Xenogen) as previously described (15).

Real time RT-PCR and Western blot analysis

Real-time quantitative PCR (qPCR) was performed with corresponding primers (Supplementary Table S1) in a TaqMan 7500 Sequence Detection System (Applied Biosystems) as described previously (18).

Western blots were performed as described previously (18) using primary antibodies specific for S100B (Abcam), full-length RAGE (FL-RAGE; Abcam), β-actin (Santa Cruz Biotechnology), S100A9 (R&D Systems), HMGBl and GAPDH (Cell Signaling Technology).

Cathepsin S assay

Primary bone marrow–derived monocytes (BMM) were harvested from WT and Ager−/− mice as described previously (19) and cultured in DMEM supplemented with 1% FBS, 100 U/mL penicillin-G, 100 μg/mL streptomycin, and 0.01 mol/L Hepes. Cathepsin S assay was performed according to the manufacturer’s instructions (AnaSpec Inc.; catalog# 72099). Briefly, 106 BMM from WT and Ager−/− mice were plated in 12-well plated for 24 hours. Cells were then treated with IFNγ (1 μg/mL), or conditioned medium (CM) from GL261or K-Luc cells. Cell lysates were collected at different time points and tested for Cathepsin S activity.

RAGE promoter activity assay

A fragment contains the 5'-flanking region (~2000 bp) of the mouse Ager (RAGE gene) was generated from mouse genomic DNA by PCR using the following primers: forward, 5'-attgtagcaggagccatagatacagct-3' and reverse, 5'-attaaggctccatctccctacggtct-3'. This product was cloned into the Nehl and HindIII sites of the pGL3-basic vector (Promega), and the generated plasmid was designated pRAGE-Luc.

Primary BMM from WT and Ager−/− mice were cultured in 6-well plates as described above. Cells were then transfected with pRAGE-Luc by lipofectamine Reagent (Invitrogen) for 36 hours and then incubated with CM from GL261.
or K-Luc cells for 12 hours. Cells were harvested by manual scraping in lysis buffer (Promega) to yield lysates that were then assayed for luciferase activity using the Luciferase Assay System (Promega). The luciferase activity was measured in a luminometer.

Vascular permeability

Mice bearing 2-week-old intracranial GL261 or K-Luc tumors were injected with TRITC-dextran 150 (Life Technology) solution (100 mg/kg i.v.). Brains were harvested after 2 hours, and fixed in paraformaldehyde for 4 hours before storage in 30% sucrose solution. Brains were then embedded in O.C.T. (optimal cutting temperature compound; Tissue-Tek), sectioned (10 μm), and baked at 37°C. Images were captured with an AX-70 fluorescent microscopy (Leica Microsystems Inc.) and analyzed by Zeiss LSM Image Browser software.

For Evans blue permeability assay, tumor-bearing mice were anesthetized and injected with Evans blue dye (100 μL of a 1% solution in 0.9% NaCl, Sigma-Aldrich) into the retro-orbital plexus. Thirty minutes later, mice were sacrificed and perfused with PBS. Brains were removed, imaged, and dried in 60°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight. The Evans blue dye was then extracted using 1 mL formamide at 55°C overnight.

Vascular density

Vascular density was calculated by measuring average vessel diameter over four × 10 fields obtained by AX-70 fluorescent microscopy (Leica Microsystems Inc.) and prepared by Zeiss LSM Image Browser software.

Flow-cytometry analysis

For in vivo staining, tumor tissue was minced and digested with trypsin for 20 minutes at 37°C. Tissue homogenate was then filtered through a 40-μm filter and prepped using fixation/permeabilization solution according to the manufacturer’s instructions (BD Pharmingen). Multiple-color FACS analyses were performed at the City of Hope FACS facility using a 3-laser CyAn immunocytometry system (Dako Cytomation), and data were analyzed using FlowJo software (TreeStar) as described previously (19). PerCP-conjugated antibodies to mouse CD45 (cat. no. 557235) were purchased from BD Pharmingen, allophycocyanin-conjugated anti-mouse CD11b (cat. no. 17-0112-82) and the primary rabbit-anti-mouse RAGE (cat. no. ab3611-100) were purchased from Abcam. Secondary goat anti-rabbit-FITC (cat. no. sc-2012) and secondary goat anti-mouse-FITC (cat. no. sc-2010) were purchased from Santa Cruz Biotechnology. TAMs (CD11b<sup>high</sup> CD45<sup>high</sup>) and microglia (CD11b<sup>high</sup> CD45<sup>low</sup>) were distinguished on the basis of previously described FACS analysis (21).

Bone marrow transplantation

Lethally irradiated recipient mice (two doses of 550 cGy every 4 hours) were injected via a tail vein with 5 × 10<sup>6</sup> bone marrow (BM) cells freshly collected from donor mice. The donor mice were euthanized and BM cells were aseptically harvested by flushing femurs with Dulbecco’s PBS (DPBS) containing 2% FBS. The samples are combined, filtered through a 40-mm nylon mesh, centrifuged, and passed through a 25 gauge needle. Recovered cells are resuspended in DPBS at a concentration of 5 × 10<sup>6</sup> viable nucleated cells per 200 μL. All animals were given autoclaved water with sulfatrim from 2 days before 2 weeks after transplantation. Six to 8 weeks after transplantation, mice were implanted intracranially with GL261 gliomas.

Immunofluorescence staining

Frozen brain sections were prepared from naive and tumor-bearing mice. Immediately after harvest, brains were fixed in paraformaldehyde for 4 hours before storage in 30% sucrose solution. Brains were embedded in O.C.T. (Tissue-Tek) and 10-μm sections were cut using cryostat (Leica Microsystems Inc.). Before immunofluorescence staining, slides were baked at 37°C and permeabilized in methanol for 15 minutes. After a 1-hour block, slides were incubated with Hypoxyprobe-F6 (1:200, Hypoxyprobe), RAGE (1:200; rabbit anti-mouse RAGE; Abcam), CD11b (1:20; rat anti-mouse; Abcam), or CD31 (1:20; rat anti-mouse; Abcam) primary antibodies for 1 hour. Slides were washed with PBS three times for 5 minutes and incubated with secondary antibody (Goat anti-rabbit Alexa Fluor 555 or Goat anti-mouse Alexa Fluor 555, 1:200 dilution Life Technologies) for another hour. Tissue sections were mounted in Vectashield mounting medium containing 4’,6’-diamidino-2-phenylindole (DAPI; Vector), imaged with AX-70 fluorescent microscopy (Leica Microsystems Inc.), and prepared by Zeiss LSM Image Browser software.

For human studies, tumor samples from 7 patients with glioblastoma and peritumoral white matter tissue from 1 patient were collected under an Institutional Review Board (IRB)-approved protocol (IRB 07074), flash-frozen, sectioned (8-μm thick), blocked with BSA (0.5% for 1 hour) before overnight incubation with primary RAGE (1:300; cat. no. 3611; Abcam), CD34 (1:100; M7165; Dako), or CD163 (1:100; VP-C374; Vector) antibodies. After washing, slides were incubated with secondary antibodies (donkey anti-mouse—A488 and anti-rabbit—A555) for 1 hour and counterstained with DAPI for 5 minutes.

Hypoxia staining

WT or Ager<sup>−/−</sup> mice bearing 2-week-old intracranial GL261 or K-Luc tumors were injected once with 100 mg/kg pimonidazole HCl (100 mg/kg in PBS, i.v.) 2 hours before tissue analysis. Tissue sections were incubated with Hypoxyprobe before imaging.

Statistical analysis

Statistical comparison in all different experimental conditions was performed with the GraphPad Prism software using two-way ANOVA or the Student t test. Survival was plotted using a Kaplan–Meier survival curve and statistical significance was determined by the log-rank (Mantel–Cox) test. A P value of less than 0.05 was considered significant.

Results

RAGE ablation prolongs survival

To evaluate the role of RAGE depletion in TME on glioma growth, two different syngeneic orthotopic glioma models...
were studied. GL261 gliomas grow as "bulky" tumors with only microscopic invasion, whereas the K-Luc model has a more invasive phenotype. In both models, animal survival was modestly, but significantly prolonged in Ager−/− mice when compared with WT controls (Fig. 1A, bottom). This improved survival correlated with slower tumor growth in the GL261, but

Figure 1. Impact of RAGE ablation in TME on glioma growth. A, Kaplan–Meier plots (top) and tumor luciferase activity (bottom) in wild-type (WT) and RAGE knockout (Ager−/−) mice implanted with either intracranial GL261 or K-Luc gliomas (n = 7–8 mice/group; MS, median survival). B, despite improvement in survival, tumor size (2 weeks after implantation) was similar in WT and Ager−/− mice. Cross sections through the largest tumor area were used for size calculations (n = 4 mice/group ± SD). C, except for increased central necrosis in the GL261 tumors in Ager−/− mice, tumor histology and invasion pattern were similar in both strains. Experimental results are representative of three separate experiments.
not the K-Luc model (Fig. 1A, bottom). Interestingly, even with improvement in animal survival, tumor sizes were similar (Fig. 1B), and except for increased necrosis in the Ager/−/− GL261 gliomas, tumor histology and invasion pattern were unchanged in the Ager/−/− mice (Fig. 1C). These findings suggested that tumor response to RAGE ablation in TME was different in each glioma model, and other factors besides tumor growth were responsible for improved survival of tumor-bearing Ager/−/− mice. Because RAGE signaling has been implicated in tumorigenesis, we next evaluated the expression of RAGE and its ligands in each model.

Expression of RAGE and its ligands in gliomas

RAGE engagement by its ligands can lead to induction of NF-κB and activation of inflammatory pathways that further upregulate RAGE expression and its ligands in a feed-forward mechanism (3). As a result, ablation of RAGE in TME may impair RAGE/ligand interactions in tumor leukocytes and abrogate tumor inflammation. Alternatively, RAGE ablation in TME may also decrease the release of soluble RAGE (sRAGE). sRAGE serves as a decoy receptor and by binding to ligands in the extracellular space antagonizes the activation of RAGE (22). Thus, by reducing sRAGE in TME, more ligands will be available to activate tumor RAGE in an autocrine fashion. To study this complex interaction in vivo, we first confirmed the expression of RAGE and its ligands in each glioma model. As expected, RAGE expression was markedly lower in the lungs and brains of Ager/−/− mice (Fig. 2A). Very low levels of RAGE staining in the Ager/−/− brains (neurons) and lungs most likely represented expression of other RAGE isoforms that were not depleted in the Ager/−/− model or may have been due to nonspecific binding of the primary RAGE antibody (Fig. 2A, bottom). Although each cell line expressed similar levels of FL-RAGE in vitro (Fig. 2B), RAGE expression in intracranial tumors was different in each mouse strain. In GL261 tumors, FL-RAGE levels were similar in WT and Ager/−/− mice (Fig. 2C, left), but in the K-Luc tumors, they were slightly higher in Ager/−/− mice (Fig. 2C, right). Considering that nuclear RAGE levels were similar in WT and Ager/−/− tumors (Fig. 2D), higher FL-RAGE protein in Ager/−/− K-Luc tumors (Fig. 2C) most likely

![Figure 2](link-to-image)
represented higher membrane-bound RAGE or sRAGE levels that were not detectable by immunohistochemistry. Because RAGE signaling can directly modulate the expression of its ligands, we next measured the levels of known glioma RAGE ligands in these models.

To assess RAGE ligands, intracranial tumors were separated from surrounding brain tissue and analyzed by Western blot analysis. In the normal brains, levels of all the ligands were slightly lower in Ager<sup>-/-</sup> mice, confirming the abrogation of the RAGE feed-forward regulation of its ligand production (Fig. 2E). Interestingly, the expression profile of RAGE ligands was different between the two glioma models; although K-Luc tumors expressed lower S100B levels than GL261 gliomas, these tumors had higher levels of proinflammatory ligands such as HMGB1 and S100A9. High levels of HMGB1 and proinflammatory S100 proteins have been implicated in tumor invasion (23), possibly accounting for the phenotypic differences between these two models. Furthermore, the expression of common RAGE ligands was similar in WT and Ager<sup>-/-</sup> mice in both tumor models. Thus, the impact of RAGE ablation on animal survival was most likely due to modulation of TME and not to alterations of RAGE signaling in tumor cells. Because inflammation has been shown to promote tumor progression in a variety of cancers (24), we next studied leukocyte trafficking in each glioma model.

**Impact of RAGE ablation on tumor inflammatory response**

To evaluate the role of RAGE in leukocyte trafficking into tumors, Ager<sup>-/-</sup> CX<sub>3</sub>CR<sub>1</sub>GFP mice were generated and implanted with each glioma model. Surprisingly, RAGE ablation in TME did not alter leukocyte trafficking or distribution in either GL261 or K-Luc tumors (Fig. 3A), and the proportion of inflammatory cells (i.e., lymphocytes, microglia, and macrophages) in each tumor type was similar in the WT and Ager<sup>-/-</sup> mice (Fig. 3B). This suggested that RAGE did not play an important role in leukocyte

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chemoattraction in these glioma models. However, when tumor cytokines were examined, both tumor models had lower expression of proinflammatory cytokines (IL6, IL1β, and TNFα) in Ager−/− mice (Fig. 3C). These findings are consistent with the proinflammatory functions of RAGE and indicate that suppression of inflammation may have abrogated tumor growth and accounted for the improved survival of glioma-bearing Ager−/− mice. Because RAGE signaling has also been implicated in tumor angiogenesis, we next evaluated the expression of angiogenic factors in each model.

**Impact of RAGE on angiogenesis**

Besides proinflammatory cytokines, tumors in Ager−/− mice also expressed lower levels of proangiogenic factors (Fig. 4A). Although MMP2, a metalloproteinase that is involved in glioma invasion and angiogenesis (25), was also expressed at lower levels in Ager−/− mice, tumor-invasive phenotypes were similar in both mouse strains (Fig. 1C). Because histologic analysis of tumors had demonstrated RAGE expression in tumor vessels (Supplementary Fig. S1), we next evaluated the vascular density in each tumor type. Although proangiogenic factors appeared to be suppressed in both glioma models in Ager−/− mice, only the GL261 tumors had an obvious vascular phenotypic change with large dilated vessels (Fig. 4B and Supplementary Fig. S2). Furthermore, GL261 tumors that typically grow as a "bulky" mass appeared to be more hypoxic in Ager−/− mice than in WT animals (Fig. 4C). The more invasive K-Luc gliomas, on the other hand, had no signs of hypoxia and exhibited normal vascular characteristics. This finding was consistent with the histologic data demonstrating central necrosis in large GL261 gliomas in Ager−/− mice (Fig. 1C).

Furthermore, poor perfusion of the GL261 gliomas in...
Ager−/− mice may have prevented the uptake of luciferin by tumors, thus accounting for the discrepancy between the tumor luciferase activity (Fig. 1A) and tumor size (Fig. 1B). In addition to changes in vascular morphology, GL261 vessels also were more permeable in Ager−/− mice (Fig. 4D and E). K-Luc vessels also appeared to be leakier in Ager−/− mice, but this higher permeability did not translate into a statistically significant increase in overall tumor permeability (Fig. 4D and E).

In summary, the impact of RAGE ablation in TME on the growth of gliomas appeared to be due to a decrease in tumor inflammation and impairment of angiogenesis. The latter phenomenon, however, was more apparent in the “bulky” GL261 glioma model that relies on angiogenesis and not the more invasive K-Luc model. To assess the significance of these findings to human tumors, we then evaluated RAGE expression in freshly harvested malignant glioma tumor samples.

RAGE expression in human malignant gliomas

RAGE expression was evaluated in seven histologically confirmed glioblastoma samples (grade 4 astrocytomas) and white matter tissue harvested from "tumor edge." All gliomas, but not cells in the "tumor edge," expressed high levels of RAGE (Fig. 5A and B). RAGE expression was also detected in both tumor vessels (CD34+ ) and TAMs (CD163+) in every glioma sample (Fig. 5C). Nearly half of TAMs expressed RAGE in each sample. Because TAMs are a prominent component of TME and play an important role in tumor angiogenesis (26), we evaluated their angiogenic properties following RAGE ablation.

Role of TAM RAGE expression on angiogenesis

As we reported previously, RAGE was expressed by both CNS microglia and myeloid-derived macrophages in GL261 gliomas (Fig. 6A; ref. 18). Furthermore, the proportion of RAGE+ microglia (50%–60%) and macrophages (~20%) in the GL261 model was very similar to the average proportion of RAGE+ TAMs in the human glioblastoma samples (50%–60%). To evaluate the impact of RAGE ablation in these cells, TAMs from intracranial GL261 gliomas were isolated and analyzed by qPCR. As expected, TAMs that originated from Ager−/− mice had lower expression of IL6 and VEGFα (Fig. 6B). Also, as compared with WT cells, BMM isolated from Ager−/− mice expressed lower levels of MMP9 (Fig. 6C) and Cathepsin S (Fig. 6D) when they were exposed to GL261 CM but not K-Luc CM. Finally, RAGE signaling was significantly attenuated in Ager−/− macrophages in response to CM from either GL261 or K-Luc cells (Fig. 6C), confirming activation of RAGE by glioma-derived factors in both models. However, in comparison with Ager−/− BMM that were

![Figure 5. RAGE expression in human glioblastomas. A, representative immunohistochemistry of a glioblastoma tumor sample (left) and tumor edge (right) from the same patient demonstrating RAGE expression in tumor and not peritumoral white matter. B, RAGE expression in another glioblastoma tissue sample demonstrating nuclear (1), membranous (2), and cytoplasmic (3) RAGE expression in tumor cells (T). C, expression of RAGE in tumor-associated vessels (CD34+, arrows) and macrophages (CD163+, arrows) in a representative glioblastoma sample. Nearly half of the TAMs expressed RAGE in every tumor.](cancerres.aacrjournals.org)
exposed to GL261 factors. Ager promoter activity was not completely abolished in the cells that were incubated with K-Luc CM, most likely due to activation of other RAGE-independent pathways by proinflammatory RAGE ligands (like HMGB1). Overall, these findings suggest that TAM RAGE activation was more important in angiogenesis in GL261 tumor, but not in the more invasive K-Luc gliomas.

TAMs in gliomas are derived from CNS microglia and circulating myeloid-derived cells such as monocytes. To evaluate the angiogenic function of RAGE in each cell type, chimeric mice were generated before tumor implantation. The feasibility of this technique was first assessed by cross transplanting BM from CX3CR1GFP and WT mice. After recovery, mice were implanted with GL261 tumors and analyzed by histochemistry and flow cytometry. Glioma macrophages were identified as CD45high CD11bhigh cells as we reported previously (21), and appeared to infiltrate into the tumors (Fig. 7A, left). In the reverse transplantation experiments, in which recipient mice were CX3CR1GFP, tumor microglia were identified as CD45intermediate CD11bhigh cells and mostly remained within the margin of the tumor (Fig. 7A, right).

To evaluate the impact of macrophage RAGE expression on tumor angiogenesis, Ager−/− mice were then used as donor BM. Total tumor VEGFα expression was significantly lower in these mice (Fig. 7B), but this VEGF decline was not as profound as when tumors were propagated in Ager−/− mice (Fig. 4A), suggesting that other myeloid-derived cells (like microglia) from WT recipient mice may have also contributed to tumor VEGFα production. To confirm this, tumor vascular density was compared in cross transplant experiments (Fig. 7C). Interestingly, only mice that lacked RAGE expression in both microglia and macrophages demonstrated large dilated vessels, confirming that RAGE expression by either tumor microglia or macrophages was sufficient to normalize angiogenesis in GL261 tumors.

Figure 6. Effect of RAGE ablation in TAMs. A, representative immunohistochemistry (left) and flow cytometry (right) of intracranial GL261 tumor in WT mice confirming RAGE expression in TAMs (left; CD11b+, arrows; right, red events). Nearly 20% of tumor macrophages (CD45high CD11bhigh) and 50% of tumor microglia (CD45intermediate CD11bhigh) expressed RAGE. B, TAM expression of IL6 and VEGFα was suppressed in Ager−/− GL261 gliomas. TAMs were isolated by Percoll gradient from intracranial GL261 gliomas 2 weeks after implantation (n = 4 mice/group ± SD); *, P < 0.05; **, P < 0.01. C, expression of MMP9 was lower in Ager−/− primary BMM after incubation with CM from GL261 cells; *, P < 0.05; **, P < 0.001. D, cathepsin S expression was modestly lower when Ager−/− BMM was incubated with IFNγ and GL261 CM, but not K-Luc CM; *, P < 0.05. E, RAGE promoter activity was measured in WT and Ager−/− BMM after incubation with CM from GL261 and K-Luc cells. Although Ager promoter activity was lower in Ager−/− BMM under both conditions, its activity was not completely abolished when cells were exposed to K-Luc CM (n = 4 ± SD); *, P < 0.01; ***, P < 0.001; ns, not significant as compared with cells transfected with control vector. Representative data from two separate experiments are shown.
Interaction of RAGE with its ligands through both autocrine and paracrine mechanisms can enhance tumor progression, invasion, and angiogenesis, and blockage of this interaction has been proposed as a potential anticancer therapy (27). In this study, we demonstrate that genetic ablation of RAGE in TME also prolonged survival of glioma-bearing mice by reducing tumor-associated inflammation and angiogenesis. The survival benefit of RAGE ablation in TME, however, was modest and its function in angiogenesis was dependent on tumor phenotype. In less invasive GL261 gliomas that grow as "bulky" tumors, RAGE ablation in TAMs reduced vascular remodeling, leading to dilated vessels, tumor hypoxia, and necrosis. In the more invasive K-Luc model, on the other hand, RAGE blockade did not have a significant impact on tumor angiogenesis. To our knowledge, this is the first report to demonstrate the proangiogenic function of RAGE signaling in tumor macrophages. Our findings also emphasize the complex role of RAGE and its ligands in gliomagenesis.

We expected RAGE ablation in TME to inhibit leukocyte trafficking into gliomas because RAGE may function as an adhesion molecule (28) and aid leukocyte recruitment by binding to CD11b (29, 30). Furthermore, RAGE has been shown to be important in S100B-mediated chemoattraction of microglia (31). In this study, however, RAGE expression did not inhibit TAM infiltration in gliomas. This finding is consistent with our recent report demonstrating that S100B-mediated promotion of leukocyte trafficking into gliomas was not dependent on RAGE expression in TME, but was mediated through upregulation of CCL2 (32). Similarly, Heijmans and colleagues (33) reported that lack of RAGE in colon polyps did not influence macrophage trafficking but instead caused a marked increase in infiltrating mast cells. These results support the presence of multiple overlapping pathways that promote TAM chemoattraction into tumors.

Although RAGE did not affect macrophage trafficking, its blockade abrogated tumor inflammation and improved survival of two phenotypically different glioma models. As a multiligand receptor, activation of RAGE can upregulate the expression of proinflammatory cytokines (4). Consistent with these reports, we noted a decrease in inflammatory cytokines in both invasive and noninvasive gliomas in Ager−/− mice. Suppression of tumor inflammation was most likely due to
inactivation of RAGE on TAMs and not to modulation of RAGE in tumor cells because levels of RAGE ligands did not significantly change in Ager−/− mice. Nevertheless, reduction of tumor inflammation was sufficient to modestly improve survival of glioma-bearing Ager−/− mice.

RAGE ablation in TME also affected angiogenesis in both glioma models. Tumors in Ager−/− mice had more permeable blood vessels and lower expression of proangiogenic factors. Circulating AGEs can contribute to vascular remodeling processes associated with inflammation and cancer (23, 34). Furthermore, RAGE is expressed by vascular smooth muscle and endothelial cells in which its activation by RAGE ligands results in their proliferation, migration, and tube formation (35–37). Also, Spiekerkoetter and colleagues (38) reported that S100A4 (a RAGE ligand) activates the MAPK pathway via RAGE, leading to the expression of MMP2, which is essential for vascular smooth muscle cell migration. Thus, in both glioma models, development of permeable vessels may have been due to direct RAGE ablation in vascular smooth muscle and endothelial cells. However, although tumor vessels were more permeable in Ager−/− mice, angiogenesis was more perturbed in the GL261 model in which vascular remodeling was diminished, resulting in poor tumor perfusion and central necrosis.

In contrast with K-Luc tumors, GL261 gliomas in Ager−/− mice were more hypoxic and necrotic due to their “bulky” phenotype and dependency on angiogenesis for growth. In these tumors, RAGE expression was necessary for vascular normalization and remodeling that was essential to tumor perfusion. The exact mechanism by which RAGE signaling in GL261 TAMS regulated angiogenesis remains unclear, but our data suggest that expression of proteinases may play a role in this process. BMM from Ager−/− mice expressed lower levels of MMP9 and Cathepsin S when incubated with GL261 CM. These proteases are secreted by macrophages and microglia in CNS tissue remodeling, and have been shown to play a role in glioma angiogenesis and invasion (25, 41, 42). Interestingly, in GL261 gliomas, RAGE expression by both resident microglia and infiltrating monocytes was important for angiogenesis in these tumors, suggesting that therapeutic strategies that only target infiltrating monocytes (and not microglia) may not be adequate in preventing angiogenesis in these tumors.

In addition to RAGE activation in TAMs, variation in the expression of RAGE ligands may have differentially affected tumor angiogenesis in response to RAGE ablation in each model. The K-Luc model expressed high levels of HMGB1 and S100A9, which enhance tumor invasion, migration, and angiogenesis (43). High levels of HMGB1 expression by K-Luc gliomas may have overcome the dependency on RAGE signaling for angiogenesis in this model. Besides RAGE, HMGB1 has been shown to bind other receptors such as TLR4 and TLR2 (44, 45). Activation of these receptors by HMGB1 that was secreted by K-Luc cells may have been responsible for stimulation of the RAGE pathway through a RAGE-independent mechanism. Perhaps inhibition of HMGB1 production in this model may be a more potent antiangiogenic strategy than inhibiting RAGE signaling.

In summary, this study demonstrates the role of TAM RAGE expression in glioma angiogenesis and highlights the diversity of RAGE signaling in these heterogeneous tumors. Although RAGE ablation in TME by itself may not be a viable treatment for malignant gliomas, targeting the interaction of RAGE ligands with their receptors may have therapeutic potential.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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