Neuro-Oncology

Thrombin-processed Ecrg4 recruits myeloid cells and induces antitumorigenic inflammation

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Background. Extensive infiltration of brain tumors by microglia and macrophages is a hallmark of tumor progression, and yet the overall tumor microenvironment is characterized by an immunosuppressive phenotype. Here we identify esophageal cancer-related gene 4 (Ecrg4) as a novel thrombin-processed monocyte chemoattractant that recruits myeloid cells, promotes their activation, and leads to a blockade of tumor progression.

Methods. Both xenograft glioma and syngeneic glioma models were used to measure orthotopic tumor progression and overall survival. Flow cytometry and immunohistochemical analyses were performed to assess myeloid cell localization, recruitment, and activation.

Results. Ecrg4 promotes monocyte recruitment and activation of microglia in a T-/B-cell–independent mechanism, which leads to a reduction in glioma tumor burden and increased survival. Mutational analysis reveals that the biological activity of Ecrg4 is dependent on a thrombin-processing site at the C-terminus, inducing monocyte invasion in vivo and in vitro. Furthermore, tumor-induced myeloid cell recruitment is impaired in *Ecrg4* knockout mice, leading to increased tumor burden and decreased survival.

Conclusions. Together, these results identify Ecrg4 as a paracrine factor that activates microglia and is chemotactic for monocytes, with potential as an antitumor therapeutic.

Keywords: Ecrg4, glioma, macrophage, microglia, monocyte.

Malignant glioma is an invasive and diffuse central nervous system tumor that is characterized by extensive immune cell infiltration.[1–4] Abundant infiltrating microglia constitute the primary resident innate immune response, which is accompanied by a mobilization of mononuclear cells, primarily inflammatory monocytes and T cells.[5] Although up to 30% of a human glioma tumor mass comprises microglia,[6] the tumor microenvironment is characterized by an overall immunosuppressive phenotype.[7] For example, microglia, as the resident macrophage of the brain, become tumor permissive, enhance glioma invasion,[2–4,7–9] and suppress pro-inflammatory antitumor surveillance functions.[1,4,10] A hallmark of their immunosuppressive phenotype is the skewing of cytokine profiles of glioma-infiltrating microglia/macrophages and lymphocytes.[1,11–13] While the specific mechanisms mediating the immunosuppressive tumor microenvironment are poorly understood, the expression of paracrine factors, including transforming growth factor β, colony stimulating factor 1, and macrophage inhibitory factor, is associated with this phenotype.[1,5,14]

Chromosome 2 open reading frame 40 (c2orf40), alternatively named esophageal cancer-related gene 4 (*Ecrg4*) is a candidate immunomodulatory factor released following brain injury, where it is expressed in normal brain neuroepithelia,[15] oligodendrocytes,[16] and astrocytes.[17] Its expression is downregulated in various tumors,[18–21] including glioma, where Ecrg4 mRNA levels are downregulated compared with matched normal brain tissue through hypermethylation of its promoter, and this pattern is more frequently observed in advanced tumor.[19,21] Previous studies have shown that elevated Ecrg4 expression levels correlate with improved survival in cancers of the esophagus, prostate, and breast, suggesting a role in tumor progression.[20,22,23] We have recently shown that Ecrg4 is highly expressed in leukocytes and encodes a highly conserved, membrane-tethered protein that is selectively shed from the cell surface following injury.[18,24] The secreted form of Ecrg4 induces neural cell senescence,[16] releases CNS hormones,[25] and stimulates the pro-inflammatory phosphorylation of p65 Rel A in the nuclear factor-kappaB (NF-κB) pathway in peritoneal macrophages,[18] suggesting its role as an immunomodulatory factor that is widely expressed on the cell surface and processed upon activation.

In this report, we establish the capacity for Ecrg4 to mediate changes in the tumor-associated innate immune response and

the effect of Ecrg4 expression on tumor burden in orthotopic brain tumor models and map a thrombin-processing site in the C-terminus of Ecrg4 that mediates the mobilization and chemotaxis of inflammatory monocytes. Thus, while Ecrg4 has been proposed to have a role in inhibiting tumor growth, a paracrine mechanism of action that accounts for its cell surface expression[24] and shedding[18] has not been addressed in tumor models. In this report, we provide evidence that a paracrine mechanism of Ecrg4 action leads to the reversal of the immunosuppressive glioma microenvironment by enhancing survival through the activation of microglia and recruitment of monocytes to the brain.

Methods

Mice

Immunodeficient Rag2 knockout (KO) mice were used for DBTRG xenograft studies,[26] and C57BL/6 mice were used for GL261 syngeneic glioma studies. CX$_3$CR1-GFP transgenic mice (B6.129P-Cx3cr1^{tm1Litt}/J, Jackson Laboratory) were used to isolate primary microglia.[27] Heterozygous Ecrg4 KO mice were purchased from the Mutant Mouse Regional Resource Center at the University of California, Davis (RIKEN cDNA 1500015O10 gene coding exon 1 was targeted by homologous recombination, generated in B6/129S5 mixed background). Ecrg4 KO mice were backcrossed to a Rag2 null background for xenografting studies to yield Ecrg4$^{-/-}$ Rag2$^{-/-}$ (Ecrg4 KO) and Ecrg4$^{+/+}$ Rag2$^{-/-}$ (Ecrg4 wild type [WT]).

Tumor Cells

Early passages of patient-derived grade IV glioma cells, DBTRG (a kind gift from Dr C. Kruse, University of California Los Angeles), were used for their highly infiltrative phenotype.[26,28,29] DBTRG cells transduced with lentivirus expressing firefly luciferase were generated as described earlier.[29] Murine GL261 glioma cells (a kind gift from Dr S. Kesari, University of California San Diego) exhibit an invasive phenotype similar to human glioma[30] and were subjected to lentiviral transduction with lenti-Ecrg4–internal ribosomal entry site (IRES)–ZsGreen or lenti-IRES-ZsGreen. B16 melanoma cells were used for subcutaneous tumor cell injections for the characterization of the effects of Ecrg4 or inflammatory cell recruitment.

Intracranial Stereotaxic Injections and Survival Analysis

Tumor cells were administered using 2 different strategies. In one, DBTRG tumor cells were mixed with lenti-Ecrg4-ZsGreen or lenti-IRES-ZsGreen and the mixture was implanted. In the other, GL261 glioma cells were transduced with lenti-Ecrg4-ZsGreen or lenti-IRES-ZsGreen, sorted 2 weeks later based on ZsGreen expression, and GL261-Ecrg4-ZsGreen or GL261-ZsGreen cells were implanted into syngeneic C57BL/6 hosts. Stereotaxic injections and in vivo bioluminescent imaging were performed as described earlier.[26] For intracranial injection of Ecrg4-derived peptides, recombinant Ecrg4$^{133-148}$ (SPYGFRHGASVNYDDY) and Ecrg4$^{37-62}$ (MLQKREAPVPTKTKVAV-DENKAKEFL) were purchased (New England Peptide), reconstituted in sterile water at 1 μg/μL concentration, and

stereotaxically injected into C57BL/6 mice. For survival studies, mice were monitored daily and the probability of survival and significance was calculated by the Kaplan–Meier method and log-rank test. All animal handling procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Immunoblotting and Interleukin-6 Enzyme-linked Immunosorbent Assay

Standard immunoblotting techniques were used with the following antibodies: anti-Ecrg4 (1:2500; HPA008546, Sigma), anti-Ecrg4 133–148 (1:1000; G-012–24, Phoenix Pharmaceuticals), and anti–β-actin (1:500; Cell Signaling Technologies). Levels of mouse interleukin (IL)-6 in the co-culture media were measured with an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

Immunohistochemistry

Standard immunohistochemistry was performed using Iba-1 (1:200; 019–19741, Wako), CD11b (1:100; 550282, Becton Dickinson [BD]), and glial fibrillary acidic protein (1:200; G3893, Sigma) as primary antibodies and Alexa Fluor–conjugated secondary antibodies (1:200; Molecular Probes). Immunostaining of tissue sections was imaged with an Olympus FluoView 1000 (ASW 1.7b) laser scanning confocal microscope equipped with 10×/0.4NA or 20×/0.7NA dry objective lenses on a BX61 microscope (Olympus).

### Plasmid Construction and PCR-Mediated Site-Directed Mutagenesis

Complementary DNAs coding for full-length (Ecrg4), full-length with R67A and K69A substitutions (FM-Ecrg4), and full-length with P131A and R132A substitutions (TM-Ecrg4) were amplified by standard PCR or PCR-mediated site-directed mutagenesis as described earlier.[24]

Monocyte Invasion Assay

Transwell cell culture chamber inserts (8 μm) were coated with 100 μL Matrigel diluted in 200 μg/mL ice-cold serum-free medium (354234, BD Bioscience). THP-1 monocytes were resuspended in serum-free media and seeded in triplicates in the upper inserts on Matrigel. Ecrg4$^{133-148}$ (10 nM) or Ecrg4$^{37-62}$ (10 nM) in 10% fetal bovine serum cell culture media was placed in the bottom of transwells. Following an 18-h incubation, cells that had invaded to the bottom side of an insert were fixed, stained with crystal violet, and counted. A total of 9 independent fields were counted per sample. Used as a positive control were THP-1 cells stimulated with 1 μg/mL lipopolysaccharide (Sigma) immediately before seeding.

Phagocytosis Assay

Carboxylate-modified fluorospheres (F-8784, Invitrogen) were added at 1:100 dilutions. Cells were incubated for 30 min at 37°C, rinsed 3 times with saline, and fixed with 2% paraformaldehyde for 10 min. Uptake of the beads was imaged with an
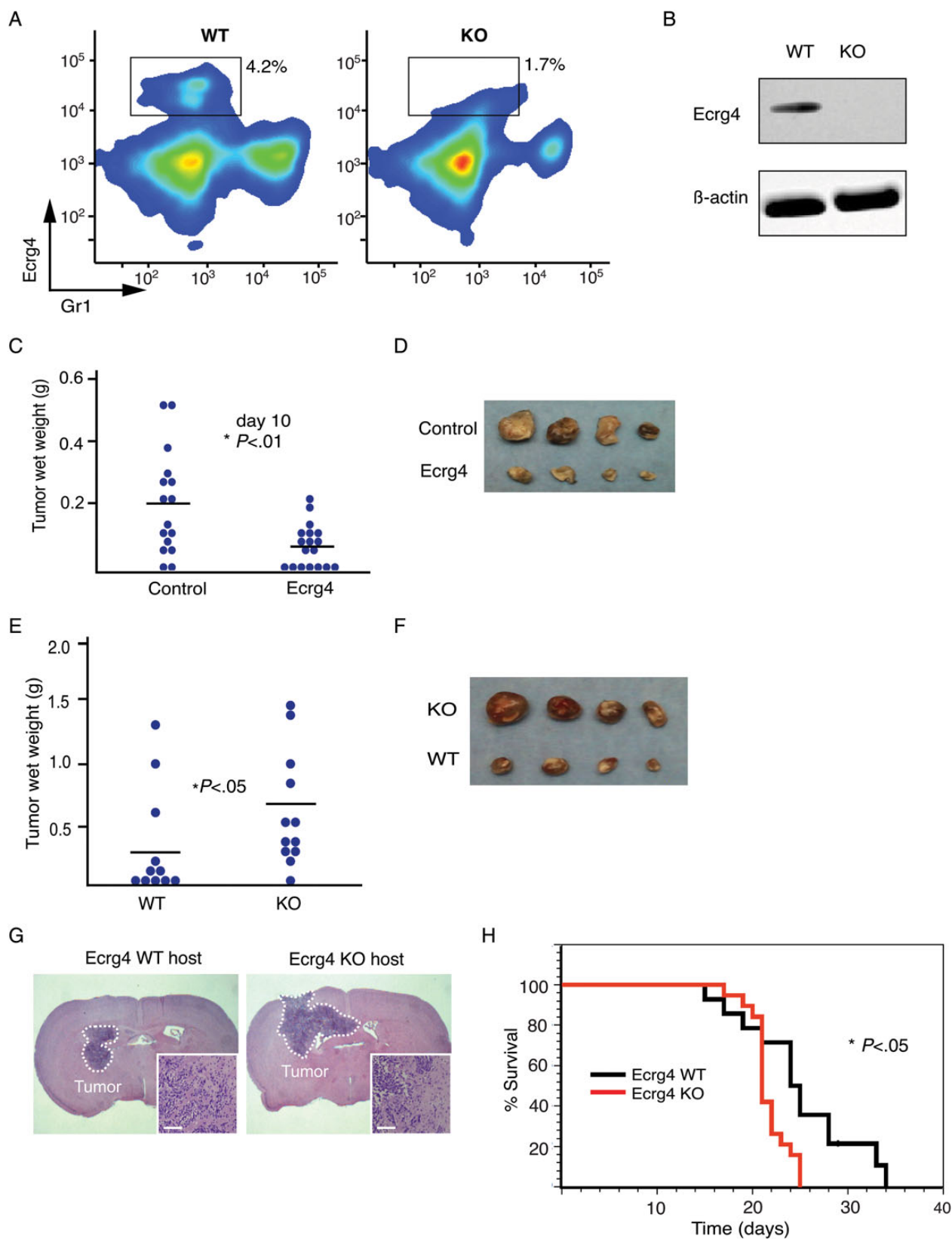
Fig. 1. Genetic knockout of *Ecrg4* demonstrates an Ecrg4-mediated regulation of tumor progression in vivo. (A) Flow analysis demonstrates surface expression of Ecrg4 in peripheral blood of Ecrg4 WT mice, which is absent in Ecrg4 KO mice. (B) Immunoblotting of lysates from peripheral

Olympus IX70 microscope, followed by analysis with Q-capture Pro software (QImaging). The percentage of phagocytic cells was determined by counting the numbers of cells that had phagocytosed more than 5 beads divided by the total number of cells. To quantify phagocytosis, at least 100 cells from 3 different fields were counted in duplicate for every measurement, and at least 4 independent experiments were performed with various mutant forms of Ecrg4. Results are presented as the percentage of control microglia cells exposed to conditioned media from cells transfected with control vector, and the baseline (8% on average) was set as 100.

Tumor Cell–Primary Microglia Co-culture Model

Primary microglia cells were isolated from 1- to 2-day-old pups as described by Saura et al.[31] DBTRG cells were transiently transfected with Ecrg4 expression plasmids (or empty vector as controls), and 48 h posttransfection an equal number of primary microglia cells were added to the cultures. Alternatively, primary microglia cells were first seeded in 24-well plates and cultured for 3 days to allow attachment to the plate, and media were substituted with conditioned media from either PC3 cells transfected with Ecrg4 expression vector or empty vector. Phagocytosis assays or IL-6 ELISA was performed at 48 h after microglia cells were added.

Flow Cytometry

Tumor-bearing brains or peptide-injected brains were harvested as described earlier[32] and incubated with the following primary antibodies: anti-CD11b-APC-Cy7 antibody (1:200; #561039, BD), anti-MHCII-FITC (1:500; #11-5321-81, eBioscience), anti-CD45-PE (1:200; 561087, clone 30-F11, BD), and anti-GR1-PE (1:500; #553128, clone RB6805, BD). Flow cytometry was performed with a BD FACSCanto, FACSAria, or Accuri instrument and data analysis performed with FlowJo software (Tree Star).

Statistical Analysis

All statistical analyses were performed using Mstat software (McArdle Laboratory for Cancer Research, University of Wisconsin). A Wilcoxon rank sum test with $P < .05$ was used to determine statistical significance. Data are presented as means $\pm$ SEM.

Results

Genetic knockout of Ecrg4 demonstrates a functional role for host-derived Ecrg4 in regulating tumor progression and survival.

Previous studies have shown that Ecrg4 is preferentially expressed on the surface of circulating human leukocytes;

therefore, to determine the functional role of Ecrg4, we analyzed Ecrg4 KO mice in a tumor model. In this first report on Ecrg4 KO mice, we observed that progeny were viable and fertile with no apparent developmental defects. We observed cell surface expression of Ecrg4 on WT leukocytes that was absent on Ecrg4 KO (Fig. 1A), consistent with immunoblotting (Fig. 1B), whereas no difference was observed in bone marrow lineages (Supplementary Fig. S1). To establish the capacity for Ecrg4 to suppress tumor progression, we performed parallel gain-of-function (Fig. 1C and D) and loss-of-function assays (Fig. 1E and F), using a subcutaneous B16 melanoma model. Adenoviral-mediated gene delivery of Ecrg4 to B16 tumors resulted in a significant Ecrg4-mediated 3-fold reduction of tumor burden by 10 days compared with control tumors (Fig. 1C and D). In contrast, B16 tumor burden increased 2-fold in Ecrg4 KO hosts at 10 days postinjection compared with WT controls (Fig. 1E and F). These findings provide a functional basis for the characterization of Ecrg4 function in orthotopic tumors such as malignant glioma in the brain. Using a mouse-derived malignant glioma with an infiltrative phenotype (GL261), we observed an increase in tumor burden and decreased survival in Ecrg4 KO mice compared with WT controls (Fig. 1G and H). Consistent with the B16 tumor model, the increase in glioma tumor burden observed in the Ecrg4 KO mice suggested that the tumor-inhibiting activity of Ecrg4 depends on the host. In contrast, gene delivery of Ecrg4 reduced tumor weight; therefore, we focused on the analysis of gain-of-function strategies to reduce tumor burden and enhance survival in malignant glioma models.

Ecrg4 Activates Iba-1$^+$ Microglia/Macrophages, Reducing Glioma Progression and Prolonging Survival

To establish the biological activity of Ecrg4 in an orthotopic glioma model, we performed gain-of-function assays and determined the effect of Ecrg4 expression on survival, tumor burden, and activation of innate immunity. Patient-derived grade IV glioma cells (DBTRG), demonstrating a highly infiltrative phenotype,[28,29,33] were subjected to lentiviral-mediated gene transduction. As previously shown with other tumor cells,[20,21] DBTRG glioma cells express low endogenous levels of Ecrg4 (Fig. 2A). In this model, bicistronic vectors expressing Ecrg4 and a fluorescent reporter were used to localize infiltrating tumor burden using confocal microscopy (see Supplementary Fig. S2), or alternatively, a luciferase reporter enabled serial, noninvasive, and quantitative measurements of glioma progression (Fig. 2).[26,34,35] Potential autocrine effects of Ecrg4 on tumor cell proliferation were addressed by in vitro analyses demonstrating no apparent effect in a panel of Ecrg4-transduced tumor cells with well-established malignant CNS phenotypes (DBTRG, GL261), as well as non-CNS malignant tumor cells (B16) (see Supplementary Fig. S3). Therefore, we

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leukocytes validated the downregulation of Ecrg4 in Ecrg4 KO mice. β-actin, loading control. (C and D) B16 melanoma cells were implanted subcutaneously with adenoviral Ecrg4 or adenoviral green fluorescent protein. Ecrg4 expression in tumors induced a reduction in tumor growth by 3-fold ($n = 18$, $P < .01$). (E and F) B16 melanoma cells were implanted subcutaneously in Ecrg4 KO or Ecrg4 WT hosts. Tumor growth was significantly enhanced in Ecrg4 KO hosts at day 10 ($n = 12$, $P < .05$) (G) Representative hematoxylin-and-eosin staining of GL261 tumors demonstrate increased tumor burden in Ecrg4 KO hosts. Infiltrate tumor margins are shown in the insert (scale bar, 100 μm). (H) Ecrg4 KO hosts demonstrated a reduced survival compared with Ecrg4 WT control ($n = 13$ WT, $n = 19$ KO; log-rank test, $P < .05$).
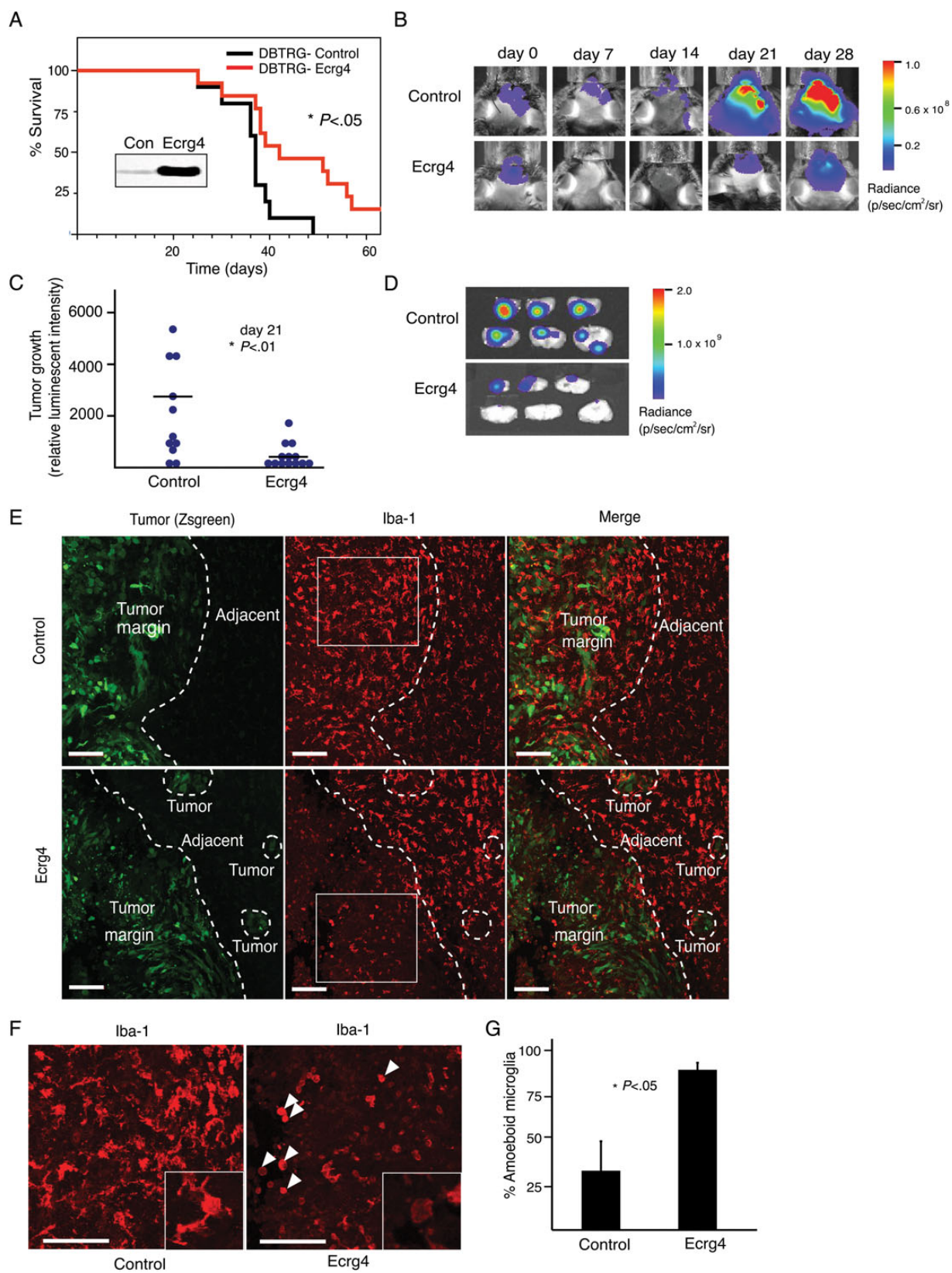
Fig. 2. Ecrg4 activates Iba-1^{+} microglia/macrophages, reducing glioma progression and prolonging survival. (A) Survival of mice with DBTRG glioma cells expressing Ecrg4 (insert) was significantly increased compared with control tumor-bearing mice ($n = 10$ controls, $n = 11$ Ecrg4

determined whether there was an effect of Ecrg4 gene expression on tumor burden in vivo. Intracranial stereotaxic injection of DBTRG glioma cells with lentivirus-Ecrg4-ZsGreen or control lentivirus was performed in immunodeficient Rag2$^{-/-}$ mice (ie, lacking T- and B-cell function)[36] to support xenograft tumor growth. Ecrg4 expression significantly increased the survival of tumor-bearing mice ($P = .02$; Fig. 2A), with noninvasive quantification demonstrating an Ecrg4-mediated 7-fold decrease in tumor burden ($P = .01$; Fig. 2B and C). This decrease in tumor burden in Ecrg4-expressing tumors was validated by bioluminescent imaging of ex vivo tumor-bearing brain slices (Fig. 2D) as described in the Methods section.

Immunohistochemical characterization of Ecrg4-expressing versus control brain tumors revealed a significant change in the tumor-associated microglia phenotype, with few changes in the astrocyte phenotype (ie, immunostaining with glial fibrillary acidic protein; see Supplementary Fig. S2). Immunostaining of glioma-bearing brain sections demonstrated a significant reorganization in the morphology of the Iba-1$^+$ microglia at the Ecrg4-glioma margins compared with adjacent area or control-glioma margins (Fig. 2E). To quantify the activation state of tumor-associated microglia, we used an established criteria on the basis of morphology (amoeboid shape vs ramified).[37] In higher-resolution images of the tumor margins (Fig. 2F), we observed that Ecrg4-expressing tumors contained >80% amoeboid microglia/macrophages, compared with control tumors with 30% of microglia/macrophages with an amoeboid morphology (Fig. 2G). The profile of microglia/macrophages in the contralateral hemisphere ("unactivated" ramified microglia) or in the hypoxic tumor core (most cells were "activated" amoeboid microglia/macrophages) in Ecrg4 versus control tumors was unchanged, suggesting that Ecrg4-mediated effects were not systemic (Supplementary Fig. S4). These findings demonstrated that Ecrg4 activates microglia in vivo and suggest a paracrine mechanism of action for Ecrg4-mediated regulation of tumor immunosurveillance.

Ecrg4 Activates CD11b$^+$CD45^{low} Microglia and Recruits CD11b$^+$CD45^{high} Monocytes, Reducing Glioma Progression and Prolonging Survival

To further investigate the capacity for Ecrg4 to mediate tumor progression in an immune-competent host and validate a paracrine biological mechanism upon host microglia/macrophages, we tested the effect of Ecrg4 in a syngeneic glioma cell (GL261) derived from a C57BL/6 genetic background that expressed low levels of endogenous Ecrg4 (Fig. 3A).[30] Following the stereotaxic implantation of GL261 glioma cells expressing Ecrg4 versus vector control, there was a significant increase in survival

(Fig. 3A), with a reduction in tumor burden as determined by histological analyses ($P = .002$; Fig. 3B). These findings with transduced GL261 glioma cells were consistent with the effects of Ecrg4 in the DBTRG xenograft glioma model (Fig. 2). Furthermore, in the GL261 model, the glioma cells were the only cell type stably expressing Ecrg4, further supporting a paracrine model of Ecrg4-mediated tumor inhibition. Our interpretation of the xenograft gliomas implanted in a Rag2$^{-/-}$ host and syngeneic mouse gliomas implanted in an immune-competent host was that the biological activity of Ecrg4 occurred through a T-/B-cell–independent mechanism. To quantify Ecrg4-mediated changes in the activation of host microglia/macrophages, cell surface expression of markers of activation (ie, major histocompatibility complex [MHC]II) was determined as previously described.[32,37] In Ecrg4-expressing GL261 tumors, we observed a significant increase of MHCII staining on CD11b$^+$CD45^{low} microglia cells compared with controls, further supporting the capacity for Ecrg4 to activate microglia/macrophages in gliomas ($P < .05$; Fig. 3C). In contrast, MHCII expression in CD11b$^+$CD45^{high} inflammatory monocytes remained unchanged (Fig. 3D).

To determine whether Ecrg4 recruits myeloid cells to the tumor microenvironment, we analyzed tumor-bearing brains by flow cytometry and quantified the infiltration of both resident and inflammatory myeloid cell populations (Fig. 3E). Ecrg4 expression led to the recruitment of CD11b$^+$CD45^{high} inflammatory monocytes to the tumor ($P < .05$; Fig. 3F), whereas no changes in the recruitment of CD11b$^+$CD45^{low} microglia or CD11b$^-$CD45^{high} lymphocytes were observed (Fig. 3F). These results demonstrated that Ecrg4 could activate microglia and mobilize myeloid cells to a brain tumor, further supporting a paracrine role for Ecrg4 in mediating tumor immunosurveillance. The chemoattractive role of Ecrg4 for myeloid cells was conserved in subcutaneous tumors as immunohistochemical analysis of the Ecrg4-expressing B16 tumors revealed a significant increase in the infiltration of CD11b$^+$ cells compared with the control B16 cells (Supplementary Fig. S5A). In addition, flow analysis indicated that CD11b$^+$ myeloid cell recruitment is impaired in Ecrg4 KO hosts compared with Ecrg4 WT hosts implanted with B16 tumor cells (Supplementary Fig. S5B–E). These results are consistent with the hypothesis of Ecrg4 as a chemoattractant for myeloid cells in tumor models.

Ecrg4 Activates Microglia Phagocytosis and Inflammation Signaling

To determine whether a paracrine mechanism of Ecrg4-mediated microglia activation could be modeled in vitro, we established co-cultures of primary microglia with glioma tumor

animals from 3 independent experiments, $P = .02$, log-rank test). (B) Representative noninvasive serial imaging of luciferase-labeled tumor cells are shown, demonstrating a reduction in tumor growth in Ecrg4 tumors. (C) Tumor growth (day 21) demonstrated a significant decrease in tumor burden in Ecrg4-treated vs control animals ($P < .01$ at day 21, $n = 9$ controls, $n = 12$ Ecrg4 animals from 4 independent experiments). (D) Ex vivo analysis (day 28) in 1-mm-thick brain sections confirmed decrease in tumor burden of Ecrg4 tumors compared with controls. (E) Representative immunostaining with an anti–Iba-1 antibody is shown to detect microglia/macrophages at tumor margins, determined by imaging of DBTRG tumor cells labeled with ZsGreen. (F) Ecrg4-mediated changes in Iba-1$^+$ microglia morphology at tumor margins (boxed area in panel [E]) is shown demonstrating an Ecrg4-induced amoeboid morphology (arrow) compared with microglia in control tumor (insert, single cell). Scale bar, 100 μm. (G) Quantification of Iba-1 immunostaining showed a significant Ecrg4-mediated increase in reactive microglia/macrophages at the tumor margin (*$P < .05$).
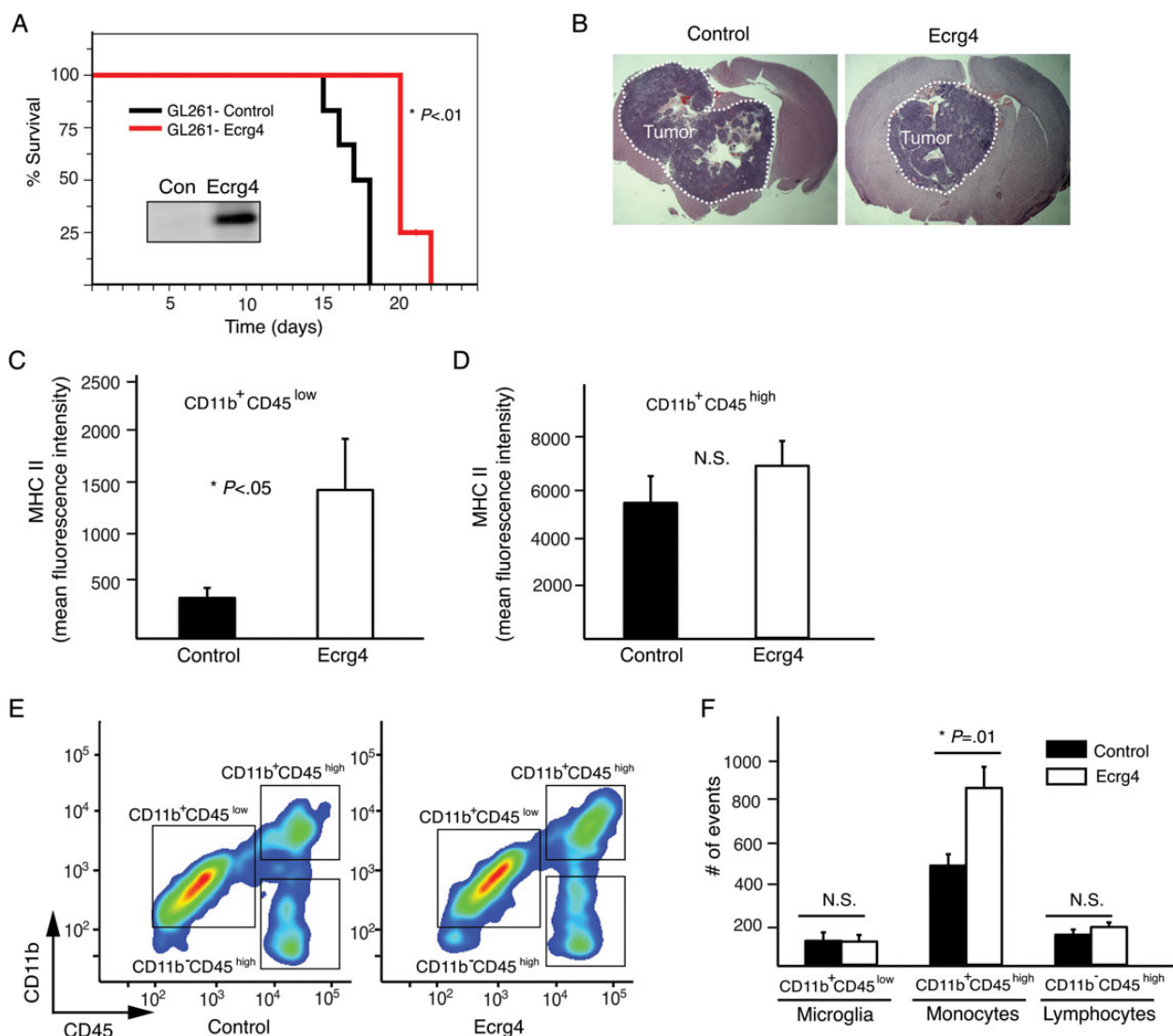
Fig. 3. Ecrg4 activates CD11b$^+$CD45^{low} microglia and recruits CD11b$^+$CD45^{high} monocytes, reducing glioma progression and prolonging survival. (A) Mice implanted with GL261-Ecrg4 (insert) demonstrated enhanced survival compared with controls ($n = 6$ controls, $n = 8$ Ecrg4; log-rank test, $P = .002$). (B) Representative hematoxylin-and-eosin staining of GL261 tumors demonstrates reduced tumor burden in Ecrg4 tumors. (C and D) Activation of tumor-associated microglia/macrophages indicated that MHCII expression was increased 4-fold in Ecrg4 tumor in CD11b$^+$CD45^{low} microglia cells ($n = 3$, $P < .05$) but not changed in CD11b$^+$CD45^{high} monocytes/macrophages ($n = 3$); N.S., not significant. (E and F) An increase in CD11b$^+$CD45^{high} cells (insert) was observed in Ecrg4 tumor, whereas recruitment of CD11b$^+$CD45^{low} microglia or CD11b$^-$CD45^{high} lymphocytes was not changed in Ecrg4 tumors compared with control tumors ($n = 6$, $P = .01$).

cells. Ecrg4-expressing versus control vector glioma tumor cells (DBTRG) were co-cultured with primary microglia. Primary microglia were isolated from mixed astroglial cultures,[31] and the purity was validated by anti–Iba-1 immunostaining and their responsiveness to lipopolysaccharide challenge. In the presence of Ecrg4, co-cultured microglia (positive for green fluorescent protein) revealed a significant increase in phagocytosis of red fluorescent beads, a hallmark of microglia activation (Fig. 4A and B). Since Ecrg4 can be expressed either in a membrane-anchored form or a processed, secreted form,[24,38] we determined whether secreted Ecrg4 affected phagocytosis. Based on mutational analysis of Ecrg4 shedding in transfected

PC3 cells,[18,24] we collected conditioned media for testing of paracrine mechanisms upon primary microglia. Following the incubation of Ecrg4 versus control conditioned media with primary microglia, we observed a significant increase in phagocytosis from the media of Ecrg4-expressing cells compared with the conditioned media from empty vector-transfected and parental cells (Fig. 4C). These observations were further supported by a significant increase in nuclear translocation of p65 (Rel A) in microglia incubated with Ecrg4-conditioned media versus control vector (Fig. 4D and E). These results were consistent with our observations in in vitro glioma-microglia co-culture studies (Fig. 4A and B), along with the Ecrg4-mediated
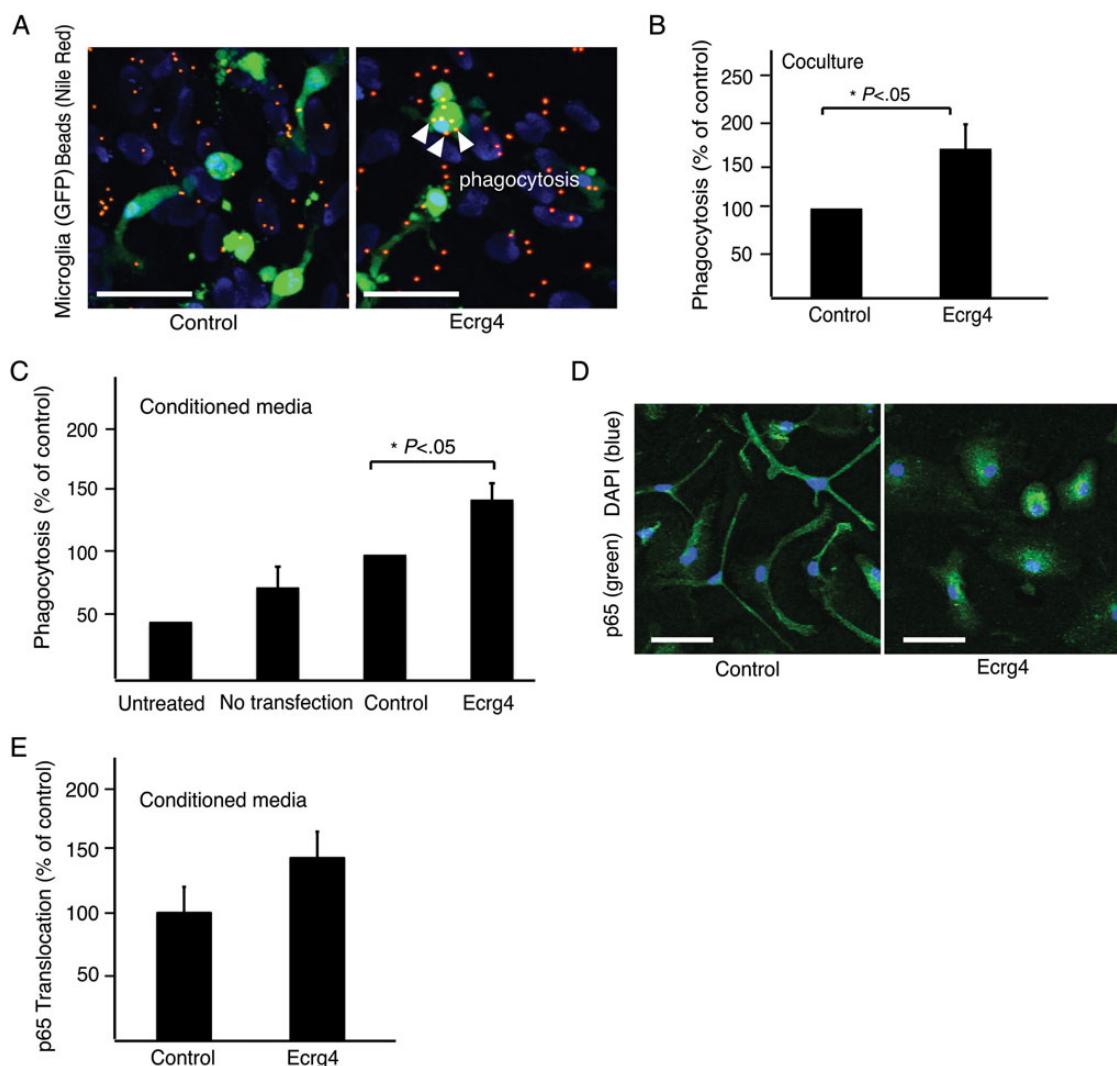
Fig. 4. Ecrg4 activates microglia phagocytosis and inflammation signaling. (A and B) GFP[+] primary microglia cells were co-cultured with DBTRG glioma cells transfected with Ecrg4 or empty vector as control. Ecrg4 expression in tumor cells induced a significant increased uptake of fluorescent microbeads (red) in microglia cells (green) as indicated by arrow showing internalized beads in microglia (4′,6′-diamidino-2-phenylindole [DAPI], blue; scale bar, 50 μm; $n = 3$ in duplicates; $P < .05$); GFP, green fluorescent protein. (C) Primary microglia were incubated with conditioned media from PC3 cells transfected with control or Ecrg4-expression vector. Untreated primary microglia or cells treated with culture media from PC3 cells (no transfection) were used as controls. Ecrg4-conditioned media activated primary microglia, as measured by phagocytosis of fluorescent beads (left) ($n = 3$ in duplicates, $P < .05$). (D) Primary microglia incubated with Ecrg4-conditioned media demonstrated nuclear translocation of p65 (Rel A), indicating activation of primary microglia by Ecrg4. Scale bar, 20 μm. (E) Quantification of panel D is shown ($n = 2$ in duplicates).

activation of microglia in vivo (Figs. 2E and F and 3C), demonstrating that shed Ecrg4 functions in a paracrine mechanism.

An Intact Thrombin Cleavage Site Is Necessary for Ecrg4 Activity

Analysis of the Ecrg4 sequence to determine a mechanism for the release of Ecrg4 from the cell surface[24] reveals putative protease processing sites for furin and thrombin[39] at the C-terminus of Ecrg4 (Fig. 5A). Incubation of recombinant Ecrg4 polypeptide with thrombin ex vivo confirmed that thrombin cleavage released a C-terminal fragment of Ecrg4 (Ecrg4[133–148]; Fig. 5B). Based on the paracrine biology of

Ecrg4 to activate microglia (Fig. 4) and the highly conserved thrombin processing sites and amino-acid sequence of Ecrg4[133–148] (Supplementary Fig. S6), we further examined the biological significance of the C-terminal Ecrg4 peptide. Conditioned media from cells expressing Ecrg4 were incubated with primary microglia, and alanine amino-acid substitutions at consensus protease cleavage sites in Ecrg4 were evaluated (ie, a furin site at amino acids 67–70, FM-Ecrg4 or thrombin site at amino acids 129–134, TM-Ecrg4) (Fig. 5C). While conditioned media from full-length Ecrg4 and the FM-Ecrg4 promoted microglia phagocytosis and induced mouse IL-6 secretion, TM-Ecrg4 induced neither microglia phagocytosis (Fig. 5D) nor IL-6 secretion (Fig. 5E). In combination, these studies
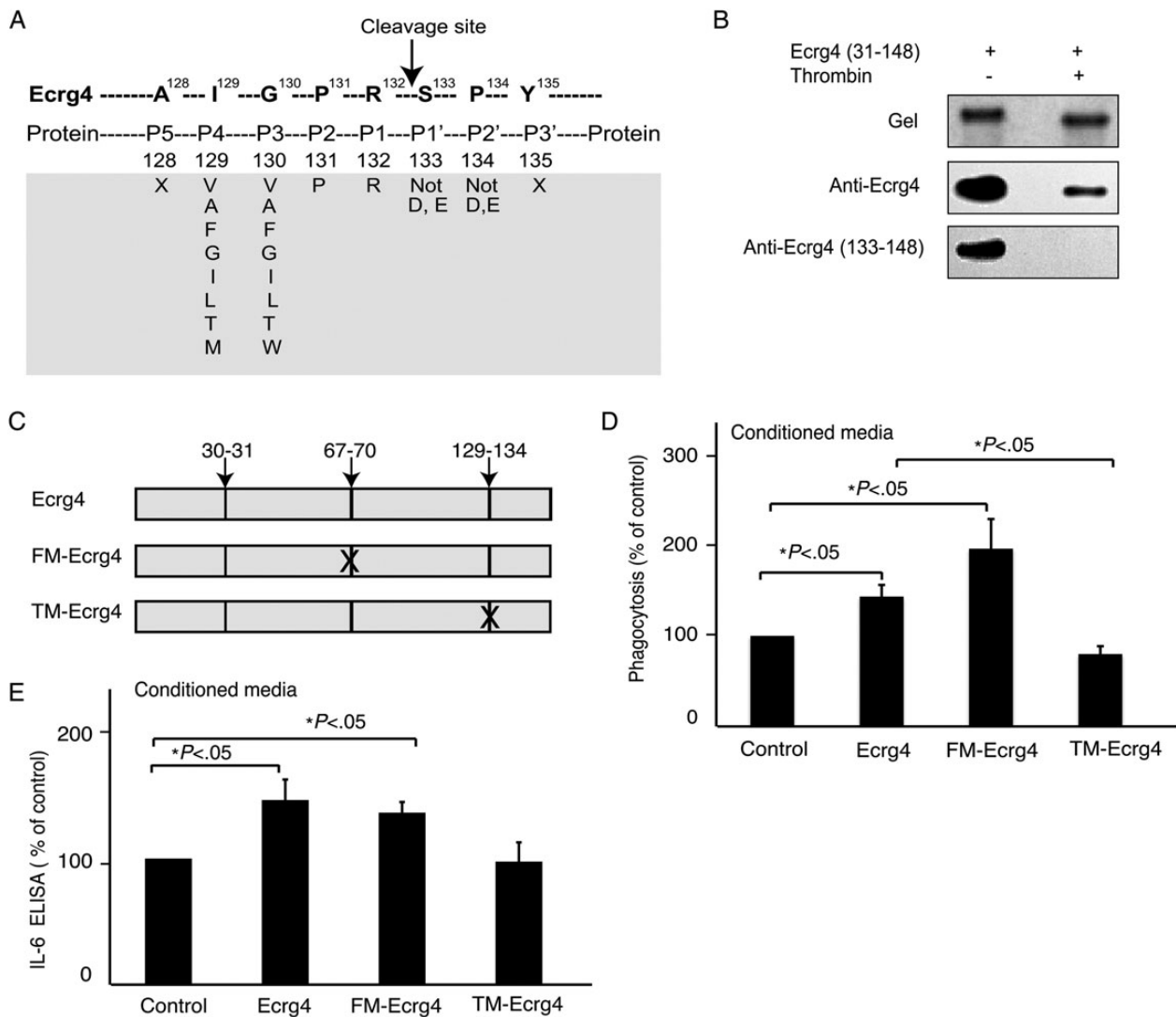
Fig. 5. An intact thrombin cleavage site is necessary for Ecrg4 activity. (A) Thrombin consensus sequence in Ecrg4 is shown in a gray box.[39] Cleavage occurs after the proline[131]-arginine[132] residue, releasing a 16 amino acid (aa) C-terminal peptide. X, any amino acid. (B) Recombinant Ecrg4$^{31-148}$ peptide (10 μg) was incubated with thrombin (10 U/mg). Thrombin processing induces a faster migrating band (Coomassie stained gel, top). These peptides were subjected to immunoblotting with anti-Ecrg4 (middle) or anti-Ecrg4 (133–148) antibody (bottom) that recognizes the C-terminal specific epitope. Thrombin-processed fragment was no longer detectable with anti-Ecrg4 (133–148) antibody (bottom). (C) Overview of putative protease cleavage sites (furin, aa 67–70 and thrombin, aa 129–134) and furin (FM-Ecrg4) and thrombin mutants (TM-Ecrg4) transfected into PC3 cells is shown. (D–E) Quantification of phagocytosis (D) or IL-6 ELISA (E) of primary microglia is shown. We observed an increase in microglia activity with conditioned media from PC3 cells transfected with WT Ecrg4 and the furin mutant (FM-Ecrg4), but not the thrombin mutant (TM-Ecrg4) ($n = 5$ in duplicates for phagocytosis, $n = 3$ in duplicates for IL-6 ELISA.) The percentage of phagocytic cells in vector control in each independent experiment (average 10%) was set to 100. (*$P < .05$).

demonstrated that Ecrg4 induced phagocytosis and cytokine secretion of primary microglia and that this biological activity of Ecrg4 required an intact thrombin cleavage site. Importantly, the mapping of the biological activity of Ecrg4 in microglia to amino acids 133–148 was consistent with our previous findings indicating that this peptide stimulates NF-κB signaling in peripheral macrophages.[18] These findings demonstrate that thrombin-processed Ecrg4 activates microglial phagocytosis and that release of IL-6 supports in vivo data that Ecrg4 mediates microglia activation.

Thrombin-Processed Ecrg4 Is Chemotactic for Monocytes In vitro and In vivo

Based on our studies demonstrating that the biological activity of Ecrg4 was dependent on proteolytic processing (Fig. 5), we determined whether thrombin-processed Ecrg4$^{133-148}$ peptide was chemotactic for myeloid cells in vitro and in vivo. To evaluate the effect of Ecrg4$^{133-148}$ on the recruitment of myeloid cells in the brain, we performed stereotaxic injection of the Ecrg4$^{133-148}$ peptide followed by dissociation of the brain and flow
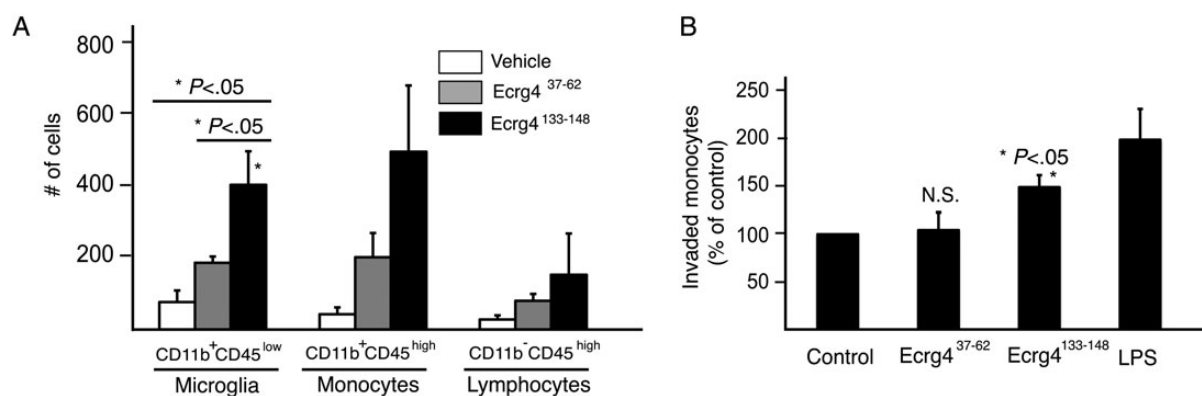
Fig. 6. Thrombin-processed Ecrg4 is chemotactic for myeloid cells. (A) Stereotaxic injection of 2 μg Ecrg4$^{133-148}$ peptide induced an increase in the number of CD11b$^+$CD45^{low} microglia at the injury site. Injection of sterile water (vehicle) or 2 μg inactive N-terminal Ecrg4$^{37-62}$ peptide was used as control ($n = 6$ each, Ecrg4$^{37-62}$ vs Ecrg4$^{133-148}$, $P < .05$). (B) THP-1 monocytes were subjected to a Matrigel invasion assay using a modified Boyden chamber. Whereas 10 nM Ecrg4$^{37-62}$ had no effect on monocyte invasion, 10 nM Ecrg4$^{133-148}$ induced a significant increase in invasion of THP-1 monocytes, demonstrating its chemotactic activity in vitro ($n = 3$ in triplicates, $P < .05$); LPS, lipopolysaccharide.

cytometry. We observed a significant Ecrg4$^{133-148}$-mediated increase in recruitment/activation of CD11b$^+$CD45^{low} microglia compared with a control, inactive N-terminal Ecrg4$^{37-62}$ peptide[18] at day 3 ($P < .05$; Fig. 6A). To establish the potential for Ecrg4 as a chemotactic factor in vitro, cultured THP-1 monocytes were subjected to an in vitro Matrigel invasion assay using a modified Boyden chamber. In the presence of thrombin-processed Ecrg4$^{133-148}$ peptide, there was a significant increase in THP-1 chemotaxis compared with the control N-terminal Ecrg4 peptide (Ecrg4$^{37-62}$), which had no effect on monocyte migration (Fig. 6B). Together, these data indicate that thrombin-processed Ecrg4$^{133-148}$ is chemotactic for myeloid cells in vitro and in vivo and that Ecrg4$^{133-148}$ mobilizes myeloid cells to mediate a pro-inflammatory role in suppressing tumor progression.

Discussion

In this study, we identify a biological activity for thrombin-processed Ecrg4 that regulates tumor inflammation through activation of microglia and recruitment of monocytes. In contrast to the mobilization of immunosuppressive immune cells that are generally associated with tumor progression, the Ecrg4-mediated activation of microglia and recruitment of anti-tumorigenic myeloid lineage cells reduce tumor progression. This unexpected mechanism of pro-inflammatory, chemokine-like activity of Ecrg4 may explain its downregulation in tumor cells and is consistent with its characterization to inhibit tumor growth.[21] However, unlike more widely studied tumor suppressor genes that are mutated in cancer cells, to this date there are no reports of genetic mutations in the c2orf40 (Ecrg4) gene associated with human cancers. Sequence variations of Ecrg4 have been detected in only a small fraction of breast tumors,[20] suggesting that the loss of the Ecrg4 gene in cancer is less attributable for its downregulation in tumors. These observations are also compatible with reports describing a downregulation of Ecrg4 gene expression in many tumors that is primarily epigenetic and mediated by promoter hyper-methylation.[21,23,40] Furthermore, we have failed to detect any

autocrine effects of Ecrg4 on tumor cell proliferation using a panel of different tumor cell lines in culture (Supplementary Fig. S3). Thus, we believe the classification of Ecrg4 as a bona fide "tumor suppressor gene" warrants further investigation.

Recent reports demonstrate that Ecrg4 is present as a membrane-anchored form in normal cells; however, following injury, infection, inflammation, and in the cultured media of transfected cells, Ecrg4 is shed from the cell surface.[24,41] While the expression of membrane-tethered Ecrg4 enhances mucosal epithelial barriers by reducing inflammatory cell infiltration,[42] here we demonstrate that thrombin-processed Ecrg4$^{133-148}$ is pro-inflammatory and mediates antitumor inflammation. The functional requirement for an intact thrombin site within Ecrg4 demonstrates that thrombin processing is required for the pro-inflammatory activity of Ecrg4 (Fig. 5). These findings are consistent with the detection of the Ecrg4 peptide in the conditioned media of transfected cells as detected by mass spectrometry,[18] in human plasma (http://www.peptideatlas.org/), and in our recent data demonstrating that the C-terminal Ecrg4$^{133-148}$ peptide activates macrophage in vitro.[18] Thus the paracrine biology of secreted Ecrg4$^{133-148}$ supports a model in which the quiescent, membrane-bound Ecrg4 is expressed as a "sentinel factor" and processed by proteases (ie, thrombin) in the tumor microenvironment. Furthermore, we show that the soluble, pro-inflammatory form of Ecrg4 is chemotactic for immature monocytes and mediates an antitumor inflammatory response. Although the exact mechanism of how secreted Ecrg4$^{133-148}$ activates microglia and recruits monocytes still warrants further investigation, our results are consistent with a hypothesis that Ecrg4 forms a complex with its receptor on innate immune cells (ie, toll-like receptor 4)[18] to recruit and activate these cells in a manner dependent on NF-κB (Fig. 4) and IL-6 (Fig. 5).

The overall immunosuppressive phenotype of brain tumors is emphasized by the various mechanisms glioma tumors use to evade immune responses of the host. For example, glioma cells secrete transforming growth factor β, inducing T-cell subsets that suppress effector T-cell function.[5,13] Similarly, microglia/macrophages that infiltrate glioma induce immune escape

and immunosuppression by the factors they secrete, such as IL-10[1], thereby promoting tumor growth. In this study, we propose a novel mechanism to reverse the immunosuppression by inducing microglia/macrophage activation and recruitment of immature monocytes at tumor margins. Whether Ecrg4 gene delivery can reprogram the "alternatively activated" M2 microglia/macrophages to "pro-inflammatory" M1 microglia/macrophages in the tumor core is not known. Moreover, the therapeutic potential of either the delivery of Ecrg4 as a gene or as a thrombin-processed peptide remains to be determined; however, our findings demonstrate that Ecrg4 is a novel therapeutic target to mediate a "pro-inflammatory" shift of the host innate response to reduce brain tumor burden.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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Conflict of interest statement. The authors declare no conflict of interest.

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