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Brain CD73 modulates interferon signaling to regulate glioblastoma invasion

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Abstract

Background: Glioblastoma (GBM) is an aggressive malignant brain-tumor that invades adjacent normal brain tissue. Unlike other solid tumors, GBM is infiltrated by various normal brain cells.

Methods: We analyzed tumor invasion in the murine GSC005 glioma model using both immunodeficient and immunocompetent mice, focusing on the role of host-intrinsic and therapeutic interferon signaling in regulating glioblastoma (GBM) invasion.

Results: In this study, we observed that mouse GBM tumor GSC005 grown in immunodeficient (RAG1-KO, NSG) mice exhibited a more invasive phenotype compared to those in immunocompetent C57BL/6J mice. Immunofluorescence staining revealed the presence of vimentin + and GFAP + cells at the tumor-border interface. Bulk mRNA-seq analysis showed that GSC005 tumors in NSG mice displayed an upregulated mesenchymal signature, characterized by epithelial-to-mesenchymal transition (EMT), and downregulation of type-I and type-II interferon signaling. Our data further suggests that host-intrinsic and therapeutic type-I interferon promotes, while type-II interferon inhibits, the GBM mesenchymal signature. CD73, a key regulator of the EMT process, was found to be upregulated in GSC005 tumors in NSG mice compared to C57BL/6J mice. Mechanistic studies revealed that type-I interferon increases CD73 expression in both tumor and stromal cells, such as tumor-associated astrocytes (mAS), while type-II interferon suppresses CD73 in mAS. Functional assays indicated that CD73 modulates both type-I and type-II interferon signaling-mediated GBM invasion.

Conclusion: These findings suggest that therapies inducing type-I or type-II interferon signaling in GBM may reciprocally regulate CD73-mediated mesenchymal transitions, impacting GBM invasion.

Key Points

- The host immune status plays a critical role in regulating glioblastoma (GBM) invasion.
- Epithelial-to-mesenchymal transition (EMT) signaling promotes, while interferon (IFN) signaling inhibits GBM invasion.
- Brain CD73 modulates both host and therapeutic IFN signaling in the context of GBM invasion.

Glioblastoma (GBM) is a highly invasive tumor that infiltrates nearby normal brain tissue but rarely metastasizes to distant organs, a feature that distinguishes it from other metastatic solid tumors. Despite extensive research, the mechanisms underlying this local invasion remain unclear. The specific tumor microenvironment of the brain, especially the interactions between tumor cells and infiltrating brain cells, such as neurons,[1] astrocytes, oligodendrocytes, and oligodendrocyte

Importance of the Study

Standard-of-care treatments and immunotherapy have been insufficient in preventing glioblastoma (GBM) invasion into adjacent healthy brain tissue, limiting overall therapeutic efficacy. Using a murine GBM model, we demonstrated that the host immune status influences tumor invasion by modulating epithelial-to-mesenchymal transition (EMT) signaling. Additionally, both host and therapeutic interferon signaling were shown to affect EMT signaling and tumor invasion. Further analysis revealed that CD73 expression in brain cells, such as astrocytes and oligodendrocytes within the tumor, regulates type-I and type-II interferon signaling related to tumor invasion. This study provides proof-of-concept for the impact of residual tumor invasion on the clinical application of novel therapeutics in GBM.

progenitor cells (OPCs), plays a crucial role in GBM invasion.

Several factors have been implicated in the regulation of GBM invasion, including hypoxia,[2] brain-tumor interactions,[1,3,4] and various signaling pathways such as TGF-β,[5] Wnt,[6] and JAK2-STAT3.[7] However, current GBM xenograft models fail to adequately replicate the invasive phenotype observed in human tumors, limiting their utility in preclinical therapeutic testing. Additionally, the role of innate and adaptive immune signaling in GBM invasion remains largely unexplored.

In this study, we first examined the infiltration of brain cells (neurons, astrocytes) in glioma tissues from patients and compared their distribution in both human and murine GBM intracranial tumor models, using immunodeficient and immunocompetent mice. We performed bulk mRNA-seq analysis of murine GBM tumors (GSC005) grown in NSG and C57BL/6J mice to identify signaling pathways that regulate immune-mediated GBM invasion. Our goal was to demonstrate how host-intrinsic and therapeutic type-I and type-II interferon signaling reciprocally influence GBM invasion via the EMT marker CD73 in both tumor cells and tumor-infiltrating astrocytes and OPCs.

Materials and Methods

Sex as a Biological Variable

Both male and female mice were included in all mouse studies.

Cells and Mice

Patients' primary GBM neurospheres, GBM12 and GBM28, were cultured in DMEM medium supplemented with 2% FBS. Murine DB7 cells were cultured in DMEM medium containing 10% FBS. Murine glioma neurospheres, GSC005, and NP cell lines were cultured in a neurosphere medium supplemented with 100 ng/mL EGF and 100 ng/mL FGF. The human astrocyte cell line, obtained as a gift from Dr. Waaqo Daddacha (Augusta University), was cultured in DMEM/F12 medium supplemented with 100 ng/mL EGF and 100 ng/mL FGF. C57BL/6, FVB/n, RAG1-KO, NSG, B6.129S7-Ifngtm1Ts/J (Ifng-knockout, stock #002287), and C57BL/6-Prf1tm1Sdz/J (Prf1-knockout, stock #002407) mice were purchased from Jackson Laboratory.

In Vivo Murine GBM Model

Human GBM neurospheres GBM12, GBM28, and GSC20 were intracranially inoculated into 6–8-week-old NSG mice. The GSC005 model was intracranially inoculated into 6–8-week-old C57BL/6 mice. NP and DB7 cells were intracranially inoculated into 6–8-week-old FVB/n mice.

Immunofluorescence Staining

Paraffin blocks were prepared from human tumor arrays (www.tissuearray.com) or from tumor-bearing mice. Following deparaffinization and antigen retrieval, tumor sections were blocked with goat serum and incubated overnight with primary antibodies targeting NeuN, GFAP, GFP, vimentin, CD140a, and CD73. After incubation, sections were treated with secondary antibodies for 1 hour. Following washes, tumor sections were mounted using a mounting medium containing DAPI and analyzed under fluorescence microscopy.

Tumor Cell Invasion Assay

FACS-sorted mouse tumor-associated astrocytes (mAS) were plated in the bottom well of a transwell (3 μm). Mock, oHSV, or OT-I CD8 T cell-treated GBM cells were plated in the top well of the transwell for 24 hours. The top well was then discarded and replaced with a new insert coated with 0.5% Matrigel. Untreated GSC005-GFP tumor cells were replated in the top well and co-cultured with mAS from GSC005-GFP tumor-bearing mice in the bottom well for an additional 16 hours. The number of GSC005-GFP cells that invaded the bottom was quantified by flow cytometry.

Flow Cytometry

For cell surface staining, cells prepared from tumor-bearing mice were washed with PBS and blocked with an Fc receptor blocker (BD Biosciences, San Jose, CA). Fluorochrome-labeled antibodies (GFAP) were obtained from BD Biosciences (Franklin Lakes, USA) and were added for a 30-minute staining period as previously described.[8] All samples were analyzed using a CytoFlex flow cytometer (Beckman Coulter, CA).

RNA Library Construction and Data Analysis

For RNA sequencing (RNA-seq), total RNA was extracted from bulk tumors, purified tumor cells, or tumor-associated astrocytes from tumor-bearing mice using the RNeasy Mini Kit (#74104, Qiagen, Germany). Poly(A)-tailed messenger RNA was enriched, and the RNA-seq library was constructed by the Georgia Cancer Center Genomics Core according to the manufacturer's instructions for the KAPA mRNA HyperPrep Kit (#KK8581, Roche Holding AG, Switzerland) and the KAPA Unique Dual-Indexed Adapter Kit (#KK8727, Roche Holding AG, Switzerland). RNA-seq data were generated using an Illumina NextSeq 550 in 75 bp paired-end mode. Raw mRNA sequence reads were pre-processed using Cutadapt (v1.15) to remove bases with quality scores < 20 and adapter sequences.

Bioinformatics Analysis

Analysis of EMT gene signatures, specifically vimentin and CD73, from the human GBM TCGA dataset, was conducted using Betastasis (www.betastasis.com) and GlioVis (http://gliovis.bioinfo.cnio.es). The spatial distribution of EMT gene signatures in GBM patients' tumor tissue was analyzed using the IVY GAP algorithm (https://glioblastoma.alleninstitute.org/). Expression of vimentin and CD73 in human GBM tumors and brain cells was assessed using data from GBMseq (www.gbmseq.org).[9]

Statistical Analysis

All quantitative results are presented as the mean ± standard deviation (SD). The statistical differences between 2 groups were evaluated using the Mann–Whitney *U* test or Student's *t*-test. For comparisons involving more than 2 groups, ANOVA was employed. Statistical analyses were performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA). A *P*-value of less than .05 was considered statistically significant.

Results

The Human GBM Xenograft in Mice Does Not Fully Recapitulate the Invasion Phenotype Observed in GBM Patients

Gliomas are known to aggressively invade adjacent normal brain tissue, which exhibits resistance to standard-of-care. Hematoxylin and Eosin (H&E) staining of normal brain tissue, as well as low- and high-grade gliomas, reveals no significant difference in cellularity between normal brain tissue and low-grade glioma (Figure 1A). However, as tumor pathological grade progresses, there is a significant increase in cellularity in high-grade gliomas compared to low-grade gliomas (Figure 1A).

Next, we investigated the infiltration of brain cells, including neurons and astrocytes, within gliomas. Our analysis indicates that both low- and high-grade gliomas are infiltrated by neurons (NeuN+) and astrocytes (GFAP+) (Figure 1B). This finding suggests that glioma cells are widely disseminated throughout brain tissue or that normal brain cells, including neurons and astrocytes, infiltrate the tumor.

To further evaluate the invasion phenotype, we assessed human GBM neurospheres (GSC20, GBM12 and GBM28) and murine GBM neurospheres (GSC005, NP and DB7) that were intracranially inoculated into the brains of immunodeficient NSG mice. and immunocompetent C57BL/6J and FVB/NJ mice, respectively Contrary to expectations, these tumors exhibited a clear boundary between tumor and brain tissue, with no significant invasion into the surrounding normal brain tissue (Figure 1C and D).

Mouse Syngeneic GBM Grown in Immunodeficient Mice Exhibited Aggressive Invasion into Adjacent Normal Brain Tissue

To analyze whether GBM invasion is regulated by host immune status, we assessed murine syngeneic GSC005 neurospheres[10] ($p53^{-/-}$-Ras^{Mut}-GFP) grown in immunocompetent C57BL/6J mice and immunocompromised RAG1-KO and NSG mice. Our findings indicate that GSC005 tumor-bearing C57BL/6J mice have a longer survival time compared to those bearing GSC005 tumors in RAG1-KO and NSG mice (Figure 2A). Furthermore, GSC005 tumors in C57BL/6J mice displayed a clear boundary between tumor and brain tissue (Figure 2B), similar to the characteristics observed in human GBM neurospheres grown in immunodeficient NSG mice (Figure 1C and D).

Conversely, GSC005 tumors grown in RAG1-KO and NSG mice demonstrated aggressive invasion into normal brain tissue, resulting in the formation of numerous small tumor islands within the tumor-bearing mice (Figure 2B). To determine whether these small tumor islands originated from tumor cells or normal brain cells, we performed staining for tumor cells using anti-GFP antibodies. The results revealed that GSC005 tumors in immunocompetent C57BL/6J mice maintained a distinct tumor-brain tissue border, while multiple small tumor islands were present at the edge of the tumor-brain tissue boundary in GSC005 tumors grown in NSG mice (Figure 2C). These findings indicate that GSC005 tumors in immunodeficient mice exhibit significant invasion into adjacent brain tissue.

We further investigated whether brain cells, including astrocytes, infiltrated GSC005 tumors grown in C57BL/6J and NSG mice. The results showed that activated astrocytes expressing high levels of GFAP + were present at the border of the tumor nodule and adjacent brain tissue in C57BL/6J tumor-bearing mice (Figure 2D). Additionally, GFAP + astrocytes were disseminated throughout the tumors in NSG mice (Figure 2D). Further staining confirmed that these activated GFAP + astrocytes were not derived from GSC005 cells (GFP+, Figure 2E).

Human and Murine GBM Grown in Mice Exhibit Distinct Expression Patterns of GFAP and Vimentin

In addition to GFAP, we further analyzed other markers that have been reported to regulate GBM invasion,[11] including vimentin, a biomarker associated with

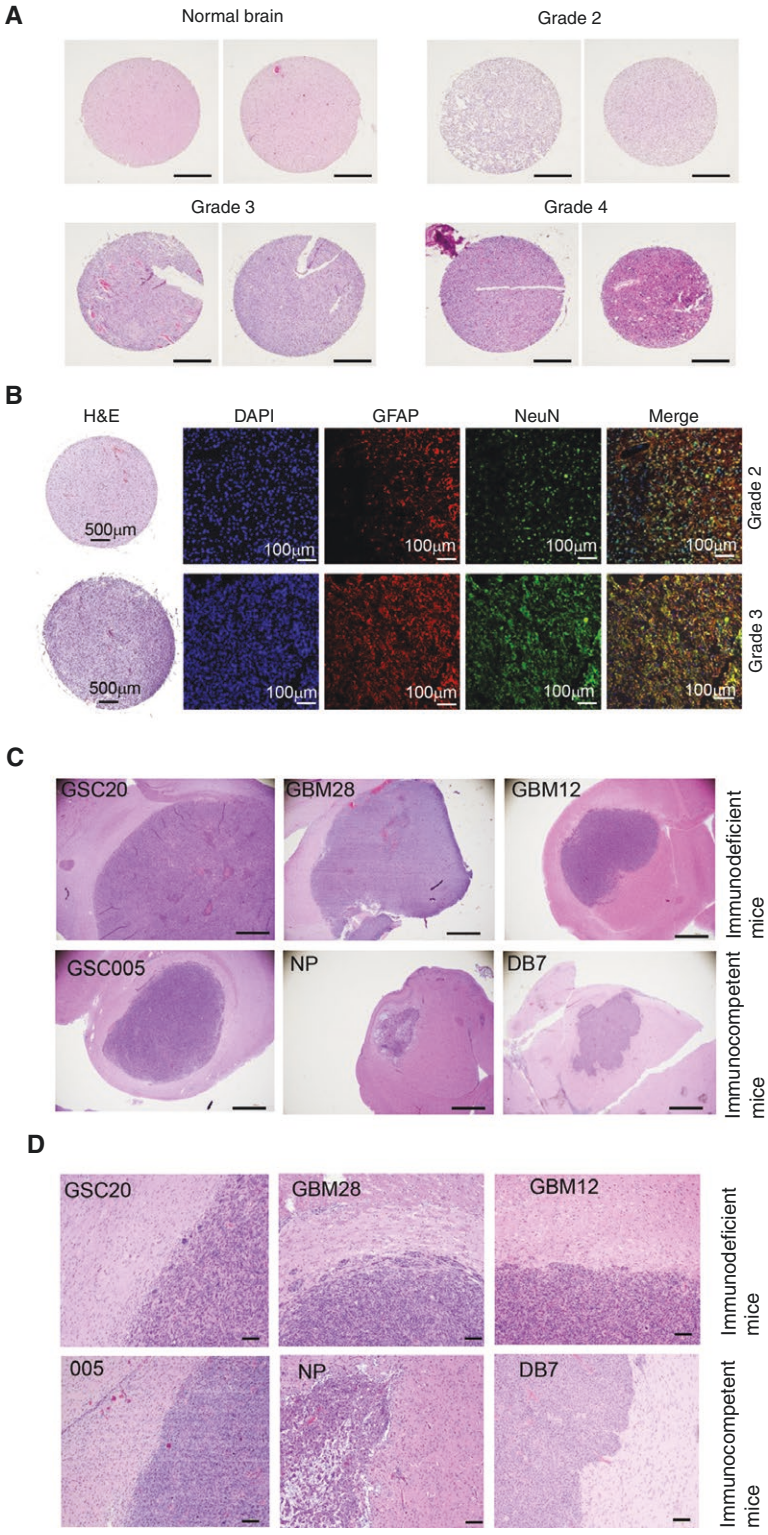

Figure 1. Infiltration of human glioma by brain cells, including neurons and astrocytes, contrasts with the clear delineation between tumors and adjacent normal tissue observed in human and murine gliomas grown in mice. (A) H&E staining of human normal brain tissue and patient-derived glioma tissue array. Scale bar = 1 mm. (B) Immunofluorescence staining of brain markers NeuN (neurons) and GFAP (astrocytes) in human normal brain and patient glioma tissue array. (C and D) Human GBM neurospheres (GSC20, GBM28, and GBM12) were intracranially inoculated into immunocompromised NSG mice, while murine GBM neurospheres (GSC005, NP, and DB7) were inoculated into immunocompetent C57BL/6J and FVB/NJ mice, respectively. Photographs depict H&E staining of intracranial tumors at low magnification (C, scale bar = 500 μm) and high magnification (D, scale bar = 100 μm). .

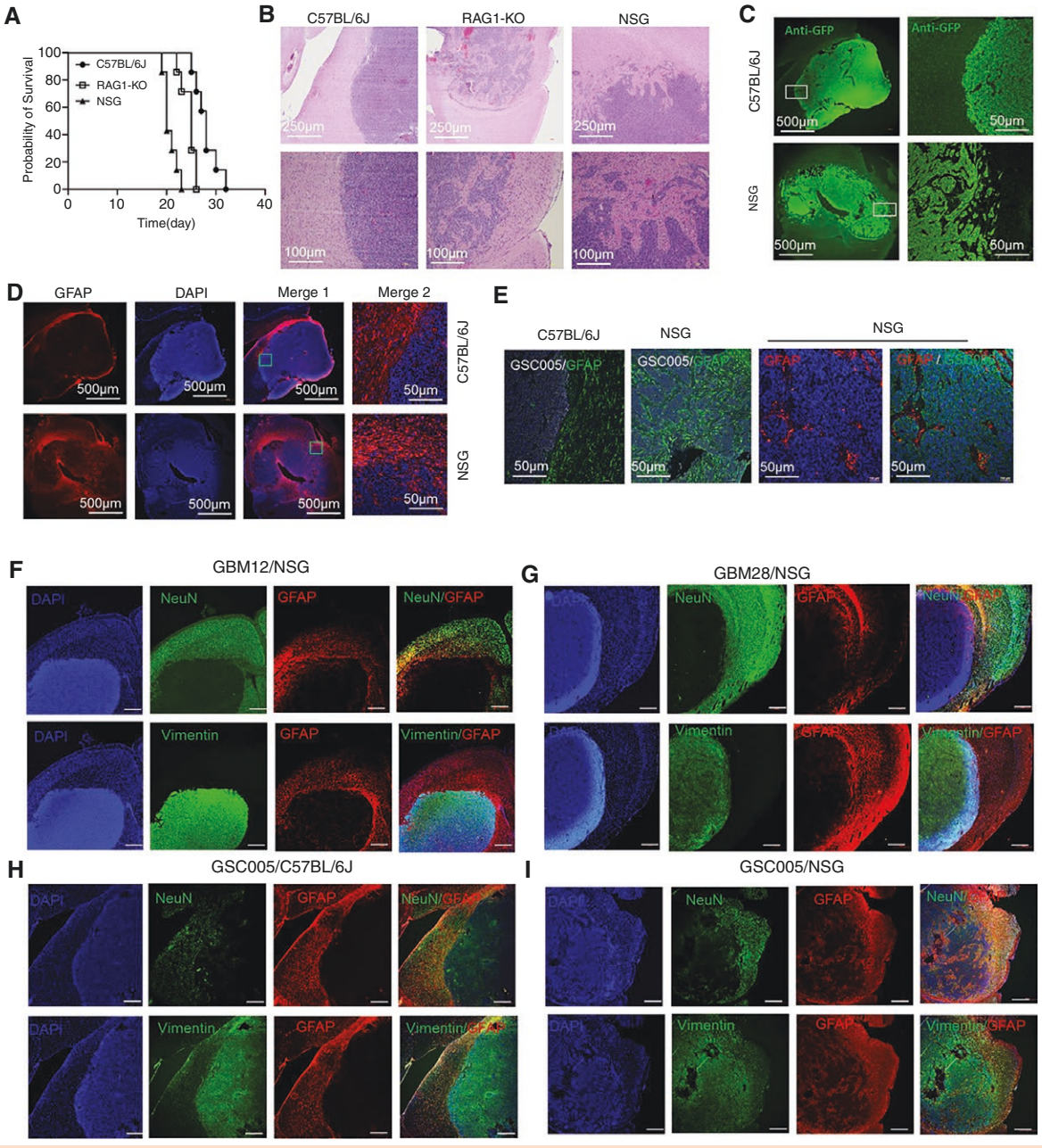

Figure 2. The host immune status regulates glioblastoma (GBM) invasion and brain cell infiltration within the tumor in both human and murine models. Mouse GBM neurospheres GSC005 were intracranially inoculated into C57BL/6J, RAG1-KO, and NSG mice. The survival of tumor-bearing mice was monitored (A, $n = 7$, $P < .05$ for RAG1-KO vs. C57BL/6J and NSG vs. C57BL/6J). In a separate cohort of tumor-bearing mice, tumors were collected 20 days post-inoculation and subjected to H&E staining, (B) and immunofluorescence staining for tumor cells (anti-GFP) (C) and astrocytes (GFAP) (D and E). Results represent one of 3 mice. (F and G) Human GBM neurospheres (GBM12 and GBM28, 1×10^5) were intracranially inoculated into immunocompromised NSG mice. Twenty days later, GBM12 and GBM28 tumors were subjected to immunofluorescence staining for NeuN, GFAP, and vimentin (scale bar = 500 μm; $n = 3$, results shown from one of 3 mice). (H and I) Murine GBM neurospheres (GSC005, 1×10^5) were inoculated into immunocompetent C57BL/6J mice and immunocompromised NSG mice. Twenty days later, GSC005 tumors were subjected to immunofluorescence staining for NeuN, GFAP, and vimentin (scale bar = 500 μm; $n = 3$, results shown from one of 3 mice).

epithelial-to-mesenchymal transition (EMT). Staining of brain markers, including NeuN (for neurons), GFAP (for astrocytes), and vimentin in human GBM12 and GBM28 neurosphere xenografts in NSG mice, revealed that neurons and astrocytes do not infiltrate the tumor nodules, maintaining a clear boundary between the brain tissue and tumor nodules in both models (Figure 2F and G). Vimentin was found to be ubiquitously expressed in tumor cells, but absent in the brain tissue of GBM12- and GBM28-bearing NSG mice (Figure 2F and G).

Next, we analyzed the expression of these markers in murine GSC005 tumors grown in C57BL/6J and NSG mice. The results indicated that neurons (NeuN+) and astrocytes (GFAP+) were primarily localized in the normal brain tissue of GSC005-bearing C57BL/6J mice (Figure 2H). Conversely, in GSC005-bearing NSG mice, neurons (NeuN+) and activated astrocytes (GFAP+) were distributed throughout the tumor (Figure 2I). Regarding vimentin expression, vimentin was highly expressed in mouse GSC005 tumors and was also detected in GFAP + astrocytes in the brains of C57BL/6J mice. Additionally, GFAP + vimentin + astrocytes were observed infiltrating both the tumor and adjacent brain tissue in NSG mice (Figure 2H and I). The difference in vimentin expression patterns between human GBM and mouse GBM in immunodeficient mice requires further investigation, particularly regarding the role of non-immune factors in regulating tumor invasion.

To determine whether this finding is applicable to other murine GBM models, we tested murine GBM NP model (NRAS, shTP53-GFP, shATRX)[12] and GL261. NP and GL261 tumors grown in NSG mice exhibited a more invasive phenotype compared to those in immunocompetent FVB/n mice (for NP cells) and C57BL/6J mice (for GL261 cell) (Supplementary Figure 1), displaying an invasion pattern similar to that observed in the GSC005 tumor. These findings indicate that host immune status regulates the expression of invasion markers in both tumor and non-tumor cells in GBM.

Vimentin, a Biomarker of EMT, Exhibits Spatial Distribution in Tumor and Brain Cells in GBM

The EMT process in GBM not only induces resistance to genotoxic drug treatments but also facilitates tumor invasion.[13] Vimentin serves as a key biomarker of the EMT process, which promotes a mesenchymal phenotype in tumors.[14] Recent studies have demonstrated that GBM can hijack brain communication for tumor invasion through the formation of tumor microtubes, with vimentin playing a significant role in this process.[1,15] Our findings indicate that vimentin is highly expressed in both murine GBM cells and GFAP + cells within the murine GSC005 syngeneic tumor model (Figure 2H and I).

Next, we investigated vimentin expression in clinical glioma patients. Immunofluorescence staining of GFAP and vimentin in normal brain tissue and various grades of glioma revealed a progressive increase in vimentin expression correlating with higher tumor pathological grades (Figure 3A). Notably, vimentin-positive cells were partially colocalized with GFAP + cells (Figure 3A), suggesting that GFAP + vimentin + cells are associated with tumor pathology and may play a role in regulating tumor invasion.

Additionally, we analyzed data from the GBM TCGA dataset and found that vimentin expression is inversely correlated with the prognosis of glioma patients (Figure 3B). Vimentin levels were significantly elevated in GBM compared to oligodendroglioma, oligoastrocytoma, and astrocytoma (Figure 3C). We also assessed the spatial distribution of EMT signaling, including vimentin, in GBM using the IVYgbm algorithm (https://glioblastoma.alleninstitute.org/). This analysis revealed that EMT signaling is predominantly activated in the hyperplastic vascular zone and microvascular zone of GBM (Figure 3D). Vimentin was found to be expressed throughout all tumor areas, including infiltrating and cellular tumors, but not in the leading edge of GBM (Figure 3D). Single-cell mRNA sequencing[9] showed that vimentin is primarily upregulated in neoplastic cells, astrocytes, vascular cells, and myeloid cells, while expression levels were lower in neurons, oligodendrocytes, and oligodendrocyte precursor cells (OPCs) (Figure 3E).

Immune-Mediated Intrinsic Interferon Signature Modulates GBM Invasion

Given the distinct tumor invasion phenotypes and varying expression patterns of GFAP and vimentin observed in GSC005 tumors grown in immunocompetent C57BL/6J and immunocompromised NSG mice, we proceeded to isolate tumor cells and GFAP + cells from GSC005-bearing C57BL/6J and NSG mice (Figure 4A) and conducted bulk mRNA sequencing analysis (Figure 4B and C). Gene Set Enrichment Analysis (GSEA) revealed that EMT signaling, which is known to regulate tumor invasion,[16] is significantly upregulated in both GSC005 + GFAP-tumor cells (Figure 4B) and GFAP + GSC005-cells (Figure 4C) from tumor-bearing NSG mice compared to those from C57BL/6J mice.

We also assessed the function of GFAP + cells isolated from GSC005 tumors in NSG and C57BL/6J mice using an in vitro transwell assay to evaluate their impact on GSC005-GFP tumor cell invasion. The results demonstrated that GFAP + cells from GSC005 tumors in NSG mice exhibited pro-invasive activity compared to those from C57BL/6J mice and U87 tumors in NSG mice (Figure 4D).

To screen for immune signaling pathways regulating GSC005 invasion in NSG and C57BL/6J mice, we performed mRNA sequencing on bulk GSC005 tumors from both mouse strains. Using the CIBERSORT algorithm, we identified monocytes/macrophages as the predominant immune cell population in both GSC005-bearing C57BL/6J and NSG mice, with a smaller proportion of T cells present in GSC005 tumors from C57BL/6J mice (Figure 4E). The host immune system, particularly interferon (IFN) signaling, plays a critical role in regulating immunogenicity and tumor MHC-I expression.[17] Bulk sequencing of GSC005 tumors revealed that MHC-I molecules are significantly downregulated in GSC005-bearing NSG mice (GSC005-NSG) compared to those from C57BL/6J mice (GSC005-B6) (Figure 4F).

GSEA analysis indicated that various immune signaling pathways, including type-I and type-II IFN signaling, are highly activated in GSC005-B6 tumors compared to GSC005-NSG tumors (Figure 4G). In contrast, pathways associated with tumorigenesis (e.g. MYC targets, G2M checkpoint, E2F targets) and tumor stromal expansion and invasion (including EMT) were significantly upregulated in GSC005-NSG tumors compared to GSC005-B6 tumors (Figure 4G). Further comparison of EMT signaling between GSC005-NSG and GSC005-B6 tumors revealed that numerous collagen-related genes (Col1a1, Col6a2, Col1a2, Col6a3, Col12a1) and integrin genes (Itga2, Itgb3, Itga5)

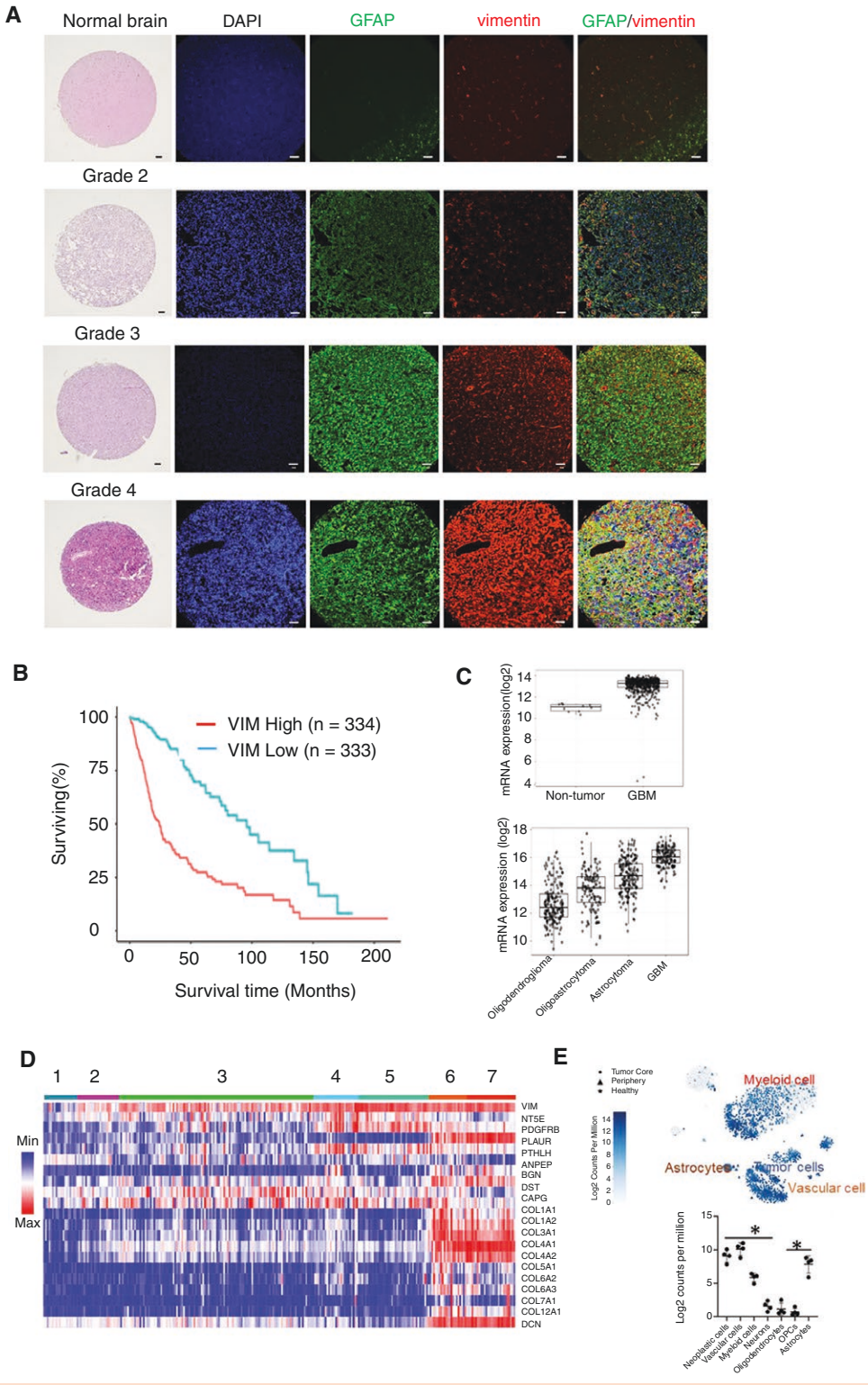

Figure 3. Spatial distribution of vimentin in tumoral and non-tumoral cells in GBM. (A) Human normal brain tissue and low- to high-grade glioma tissue array subjected to immunofluorescence staining for GFAP and vimentin (scale bar = 100 μm). (B) Kaplan-Meier survival curve comparing vimentin (VIM) low- and high-patients from the TCGA GBMLGG dataset (p = .003; Wilcoxon test). (C) Vimentin expression in GBM and other brain tumors, including oligodendroglioma, oligoastrocytoma, and astrocytoma. (D) Distribution of vimentin and other EMT markers in the tumor core, leading edge, and infiltrating tumor from the TCGA GBM dataset, categorized as follows: 1. Leading edge; 2. Infiltrating tumor; 3. Cellular tumor; 4. Perinecrotic zone; 5. Necrotic zone; 6. Hyperplastic vascular zone; 7. Microvascular zone. (E) Vimentin expression in different cell populations analyzed from scRNA-seq data from 4 GBM patients, primarily in tumor cells, astrocytes, myeloid cells, and vascular cells.

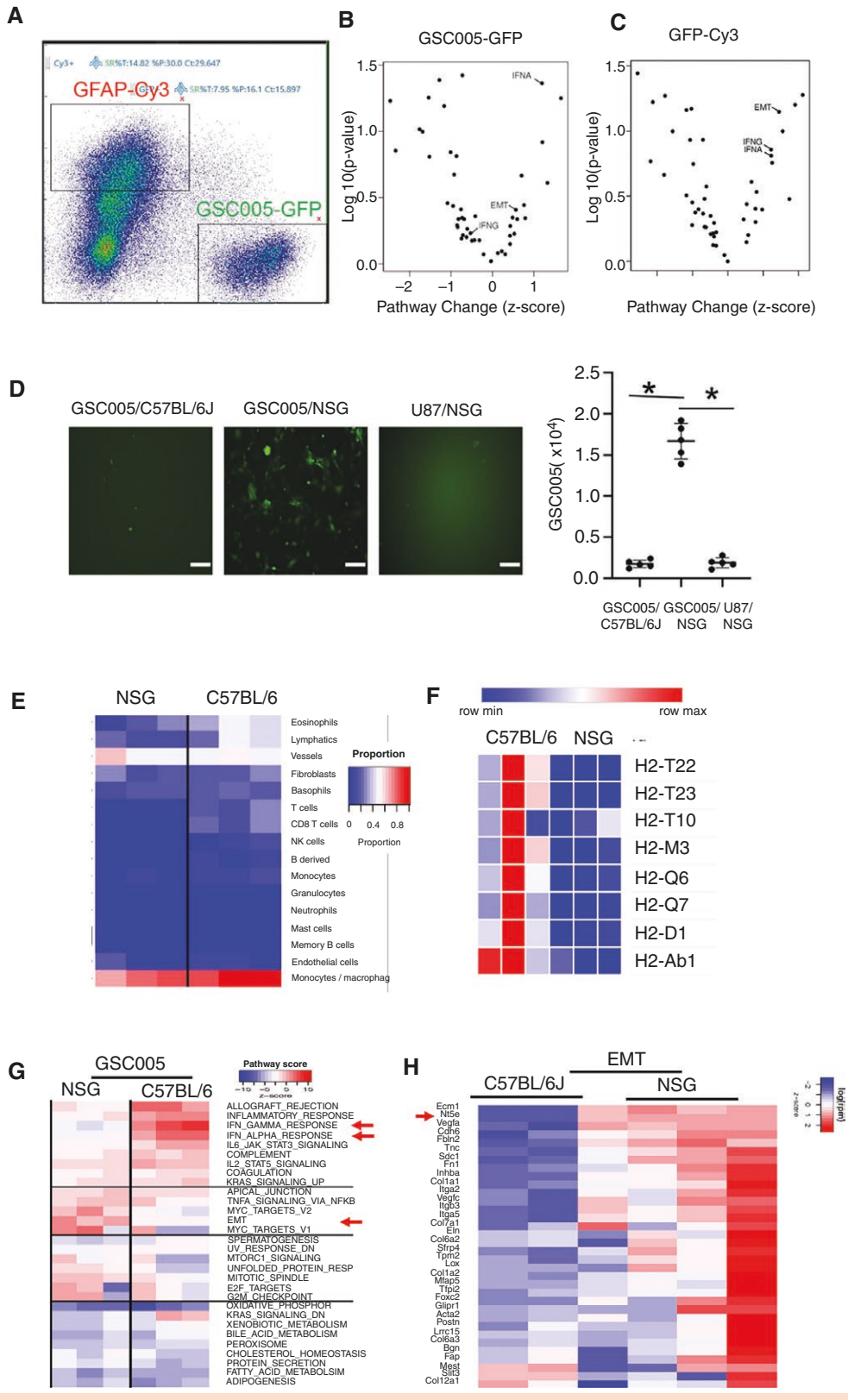

Figure 4. Host intrinsic interferon signaling modulates EMT gene signatures and tumor invasion in GBM. (A and B) mRNA-seq analysis of GFAP + cells and GSC005 tumor cells derived from tumors grown in C57BL/6J and NSG mice. GFAP + cells and GSC005 tumor cells from day 20 were FACS-sorted (A) and analyzed via mRNA-seq (B and C). Pathway enrichment analysis revealed significant upregulation of EMT signaling pathways and downregulation of type-I and type-II IFN signaling in both GSC005 tumor cells (B) and GFAP + cells (C) from NSG mice compared to C57BL/6J mice. (D) Transwell assay demonstrating GSC005 cell migration toward GFAP + cells isolated from tumor-bearing C57BL/6J and NSG

mice. GFAP + cells from GSC005-bearing C57BL/6J and NSG mice, as well as U87-bearing NSG mice, were FACS-sorted and plated in the bottom well of the transwell plate with Matrigel in the top well. 2×10^5 GSC005 tumor cells were plated in the top well, and migration to the bottom well was quantified (scale bar = 100 μm; $n = 5$, $^*P < .05$). (E to H) Bulk mRNA-seq analysis of GSC005 tumors grown in NSG and C57BL/6J mice. Tumors were harvested on day 20 for mRNA-seq analysis. The CIBERSORT algorithm was employed to analyze immune cell infiltration differences (E). (F) Heatmap analysis of MHC-I molecules in GSC005 tumors from C57BL/6J and NSG mice. (G) GSEA analysis of signaling pathways in GSC005 tumors from C57BL/6J and NSG mice. (H) Heatmap of EMT signaling pathways in GSC005 tumors from C57BL/6J and NSG mice ($n = 3$).

are highly expressed in GSC005-NSG tumors relative to GSC005-B6 tumors (Figure 4H).

Notably, Nt5e, which encodes CD73 and is involved in regulating immune responses,[18] was also found to be highly upregulated in the EMT pathway in GSC005-NSG tumors compared to GSC005-B6 tumors (Figure 4H). These results suggest that CD73 may play a role in tumor invasion in immunodeficient mice.

Interferon Signaling Regulates Brain CD73 During GBM Mesenchymal Transition

To further investigate whether the differences in GSC005 invasion between NSG and C57BL/6J mice are attributable to dysregulated interferon (IFN) signaling and EMT signaling, including CD73 expression, we first evaluated CD73 expression in tumor and brain cells from GBM patient tumor samples using single-cell RNA sequencing (scRNA-seq) analysis.[9] Our findings revealed that CD73 is predominantly expressed in malignant tumor cells, astrocytes, oligodendrocyte precursor cells (OPCs), as well as in certain myeloid and vascular cells (Figure 5A and B). Given that astrocytes represent a major cell population in human GBM that expresses CD73 (Figure 5A and B), we assessed the impact of IFN on CD73 and other EMT genes (vimentin and Grem1[19]) in astrocytes in vitro. Our results demonstrated that the addition of type-I IFN-β significantly increases the expression of EMT-related genes CD73, vimentin, and Grem1 in astrocytes, while type-II IFN-γ downregulates these genes (Figure 5C). This indicates that type-I and type-II signaling may exert opposing effects on the regulation of astrocyte function in GBM invasion.

To determine whether defective type-I and type-II IFN signaling influence GSC005 tumor growth and invasion, we conducted experiments using IFN-γ knockout (Ifng-ko) mice. The results indicated that impaired IFN-γ signaling promotes tumor growth, as demonstrated by survival curves (Figure 5D), and enhances tumor invasion (Figure 5E). Immunofluorescence staining revealed a higher infiltration of GFAP + cells into the tumors (Figure 5F), and CD73 + cells were observed distributed around GFAP + cells in GSC005 tumors grown in Ifng-KO mice compared to those grown in C57BL/6J mice (Figure 5G). Additionally, we stained PDGFRA (CD140a) and CD73 and found that some OPCs (CD140a+) were also CD73 positive in both wild-type and IFN-γ knockout mice (Figure 5H). These results suggest that IFN-γ signaling may regulate GBM growth and invasion while influencing CD73 expression in various cell types, including astrocytes and OPCs, within the GBM tumor microenvironment.

CD73 Regulates Therapeutic Type-I and Type-II Interferon Signaling of Astrocytes in GBM Invasion

Host intrinsic interferon (IFN) signaling not only regulates GBM growth and invasion, but many therapeutic approaches can also induce systemic or localized activation of type-I and/or type-II IFN signaling. In this study, we utilized intra-tumoral injection of oncolytic herpes simplex virus (oHSV)[8,20] to stimulate type-I IFN activation in the tumor microenvironment and employed cytotoxic immune cells (OT-I T cells) to activate type-II IFN (IFN-γ) within the GBM tumor microenvironment. We assessed the effects of these treatments on tumor-infiltrated brain cells, specifically astrocytes, in vitro (Figure 6A).

GSC005-OVA cells were either infected with oHSV or co-cultured with OT-I T cells for one hour, after which they were placed on the top of a transwell plate (3 μm) with mouse astrocytes (mAS) derived from GSC005-bearing mice, which were cultured in the bottom for 24 hours. The levels of IFN-β (Figure 6B) and IFN-γ (Figure 6C) were quantified using ELISA. The results demonstrated that oHSV infection of GSC005-OVA significantly increased IFN-β levels in co-cultured astrocytes, while OT-I T cell-mediated cytotoxicity led to a significant increase in IFN-γ production in these astrocytes (Figure 6B and C). Further analysis revealed that oHSV infection induced an increase in the expression of EMT-related genes, including vimentin (VIM) (Figure 6D) and CD73 (Figure 6E) in astrocytes, an effect that could be reversed by the addition of an anti-IFN-β antibody. Conversely, OT-I T cell-mediated IFN-γ suppressed the expression of both VIM (Figure 6D) and CD73 (Figure 6E) in co-cultured astrocytes. These findings indicate that therapeutic type-I and type-II IFN signaling have opposing effects on the EMT pathway in tumor-associated astrocytes.

To investigate whether CD73 plays a direct role in regulating IFN-educated mAS and their impact on GBM invasion, mAS from GSC005-bearing mice were first transfected with CD73 siRNA and then co-cultured with oHSV or OT-I T cell-treated GSC005-OVA in a transwell assay. The invasion of GSC005 toward these mAS was subsequently analyzed on a Matrigel transwell plate (Figure 6F). The results indicated that type-I IFN-educated (oHSV) mAS enhanced GSC005 invasion, an effect that was abolished in CD73 knockdown (KD) mAS. In contrast, type-II IFN-educated (OT-I T cell) mAS suppressed GSC005 invasion, with invasion further decreased in CD73 KD mAS (Figure 6G). We repeated the experiment using human GBM neurospheres GBM12 and human astrocytes and showed similar results (Figure 6H).

To further investigate the role of CD73 in regulating immune-mediated GBM invasion, 005-bearing C57BL/6J

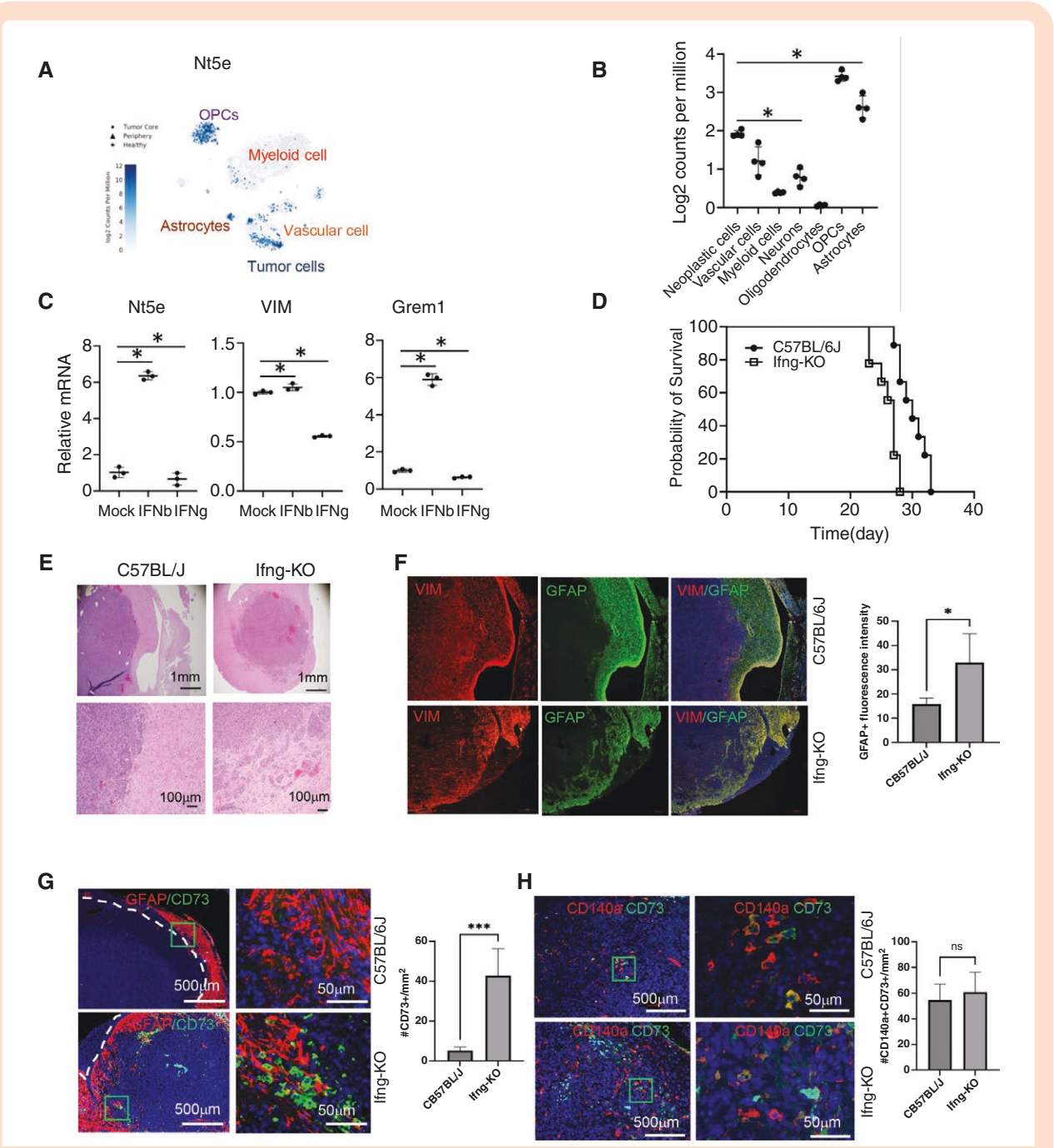

Figure 5. Regulation of GBM invasion by host interferon signaling through modification of the EMT gene signature, including CD73. (A and B) CD73 expression in different cell populations from scRNA-seq data from 4 GBM patients, showing primary expression in tumor cells, oligodendrocyte precursor cells (OPCs), astrocytes, neurons, and vascular cells. (C) Expression of EMT signature genes CD73, vimentin, and Grem1 in mouse tumor-associated astrocytes stimulated with type-I IFN (IFNb) or type-II IFN (IFNg) for 16 hours, detected by qRT-PCR ($n = 3$, $^*p < .05$). (D) Survival comparison of GSC005 tumor-bearing Ifng-KO and WT mice. (E–H) H&E (E), GFAP and vimentin (F), CD73 and GFAP (G), and CD73 and CD140a (H) staining and quantification of GSC005 tumors grown in Ifng-KO and WT mice on day 20 ($n = 3$).

mice were treated with either a CD73 blockade antibody or isotype control rat IgG. The results demonstrated that CD73 blockade increased the levels of type-I IFN (IFNb) and type-II IFN (IFNg), while decreasing vimentin (VIM) expression in tumor-infiltrated mAS compared to those from isotype-treated mice (Supplementary Figure 3a). Additionally, mAS isolated from CD73 blockade-treated mice exhibited inhibited 005 tumor invasion in vitro (Supplementary Figure 3b).

These findings suggest that CD73 plays a crucial role in regulating the activity of type-I and type-II IFN-educated mAS in GBM invasion.

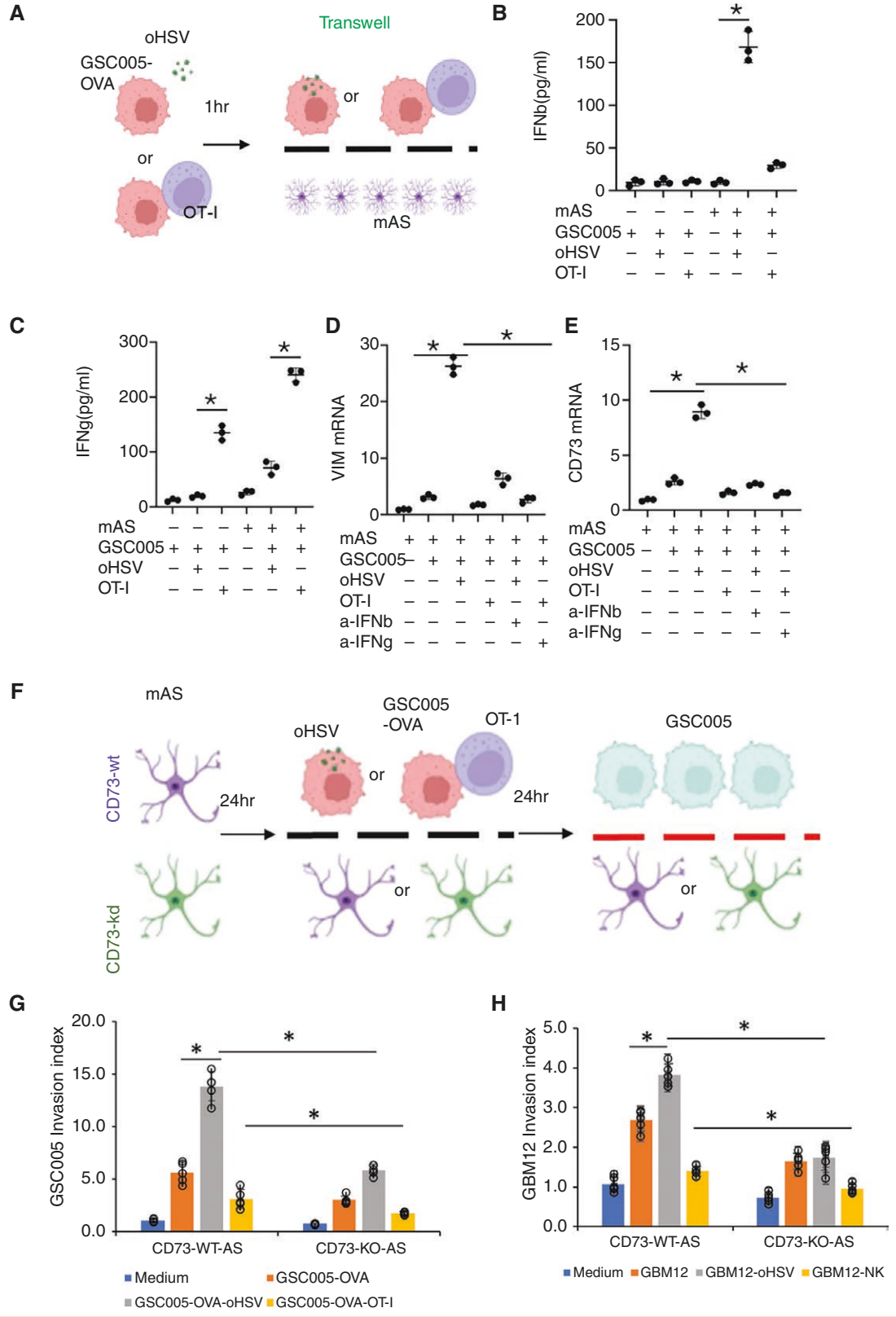

Figure 6. CD73 regulates therapeutic type-I and type-II interferon signaling in astrocytes affecting GBM invasion. (A to E) Diagram of therapeutic type-I and type-II IFN signaling on mouse astrocytes (mAS) isolated from GSC005-bearing mice in the transwell assay. GSC005-OVA cells were infected with oHSV (MOI = 0.02) for 1 hour or co-cultured with OT-I T cells for 1 hour, and subsequently transferred to the top well of the transwell plate with mAS in the bottom well. After 16 hours of co-culture, culture supernatants from the bottom well were collected for ELISA

assays of IFNb and IFNg (B and C). mAS from the bottom well were harvested for qRT-PCR analysis of VIM (D) and CD73 (E) ($n = 3$, $^*p < .05$). (F and G) Diagram of the mouse GSC005 tumor cell invasion assay (F). mAS cells were isolated from GSC005-bearing mice and plated in the bottom well of the transwell plate. Scramble siRNA or CD73 siRNA were transfected into mAS for 24 hours. oHSV or OT-I T cell-educated GSC005-OVA cells were then plated in the top well for an additional 24 hours. The insert was subsequently discarded and replaced with a new insert coated with Matrigel, followed by plating GSC005 (GFP+) tumor cells for 16 hours. The number of migrated GSC005 (GFP+) cells to mAS was quantified (G) ($n = 5$, $^*p < .05$). (H) The experiment was repeated using a human astrocyte cell line co-cultured with human GBM12-mcherry neurospheres infected with oHSV or co-cultured with human NK cells in the transwell setup. The effects of educated human astrocytes on GBM12 invasion were assessed ($n = 5$, $^*p < .05$).

Discussion

In this study, we identified that brain cells, including neurons and astrocytes, infiltrate patients' GBM tumors and express invasion-related molecules such as vimentin and CD73. However, the invasive phenotype and brain cell infiltration observed in human GBM are not fully recapitulated in murine GBM models, particularly those grown in immunocompetent mice. The tumors in immunodeficient mice exhibited partial invasive capacity, highlighting the importance of the immune environment in GBM invasion. Mechanistically, type-I and type-II interferon (IFN) signaling were found to reciprocally modulate tumor invasion through the epithelial-mesenchymal transition (EMT) process. CD73 expression in non-tumoral brain cells, such as astrocytes, oligodendrocytes, and OPCs, plays a critical role in regulating IFN-mediated tumor invasion.

Patients with GBM typically have poor prognosis, even with standard-of-care treatments. One major reason for this is the aggressive nature of GBM invasion, which often leads to therapy resistance. While therapeutics can reduce the bulk of the tumor, most do not effectively target invasive regions of the tumor, which have maximum contact with normal brain tissue. GBM invasion is driven by multiple factors, including hypoxia,[2] brain-tumor interaction,[1,3,4] and immunosuppressive cells. The unique GBM tumor microenvironment (TME), distinct from other solid tumors, contributes to this aggressive behavior. Despite its invasive nature, GBM rarely metastasizes to other organs due to the presence of the blood-brain barrier (BBB), which restricts metastasis through the blood or lymphatic systems.

Recent studies suggest that interactions between GBM cells and normal brain cells, including neurons and astrocytes, promote GBM progression. For example, Deneen et al.[1] demonstrated that remote brain stimulation could enhance glioma progression through SEMA4F-mediated mechanisms. Astrocytes, which make up approximately 50% of the brain's cellular population, play pivotal roles in both physiological and pathological processes. In normal physiological conditions, different subpopulations of astrocytes maintain key functions, including BBB integrity, metabolism, and brain synaptic activity.[4] Under pathological conditions, such as GBM, astrocytes undergo a transformation from a resting state to an activated state, known as astrogliosis.[4] During GBM development, the crosstalk between glioma cells and astrocytes can activate several signaling pathways, including NF-κB,[21] Sonic hedgehog,[22] and p53,[23] which in turn promote tumor growth and invasion. Tumor-associated astrocytes also contribute to the formation of an immunosuppressive TME, aiding tumor progression.[24,25]

GBM invasion is closely linked to the EMT process, although GBM cells typically lack the key EMT marker E-cadherin.[26] Several factors, including hypoxia,[27] TGF-β,[28] and immunosuppressive myeloid cells,[29] contribute to the mesenchymal transition of GBM cells, promoting their invasive potential. In clinical observations, the proneural subtype of GBM, characterized by a high mesenchymal nature, is highly invasive and associated with poor prognosis. Non-mesenchymal GBM subtypes can also acquire mesenchymal features following chemotherapy and radiotherapy. The classic EMT transcription factors, such as Twist,[30] Snail,[31] Wnt,[32] and NOTCH,[33] contribute to GBM invasion, with vimentin serving as a key biomarker for EMT. Beyond transcriptional regulation, vimentin expression is also influenced by epigenetic factors, including DNA methylation and non-coding RNAs. Our study further demonstrates that immune signaling can modulate the EMT process and, consequently, tumor invasion. Notably, there is a discrepancy in vimentin expression between patient samples and tumor xenografts in mice. The detailed mechanisms underlying EMT regulation of GBM invasion warrant further investigation.

The role of the immune system in regulating GBM invasion remains an area of active investigation. The brain's unique immunosuppressive environment, which supports GBM growth, is further reinforced by standard treatments like chemotherapy and dexamethasone, which can upregulate immune checkpoint inhibitors (e.g. CTLA-4) in tumor-infiltrating T cells, promoting immunosuppression and facilitating GBM growth.[34–36] Despite this, the precise mechanisms by which immune cells interact with astrocytes in the tumor parenchyma to regulate GBM invasion into adjacent normal brain tissue are not well understood. Previous research has shown that host intrinsic type-II IFN-γ signaling[17] can regulate tumorigenesis and GBM progression. Our study adds to this by showing that type-I IFN promotes EMT signaling, while type-II IFN inhibits it. Therapeutics that induce either type-I or type-II IFN responses can, therefore, indirectly affect tumor invasion. For example, intra-tumoral injection of oHSV predominantly triggers anti-viral type-I IFN responses, whereas adoptive T-cell transfer therapies elicit strong type-II IFN responses, which are often associated with immune cell cytotoxicity. However, many other factors involved in the modulation of the EMT process remain to be elucidated.

Given that Ifn-knockout mice exhibit increased GBM invasion, we also explored the role of another host cytotoxic molecule, perforin, in glioma development using perforin-knockout (Prf1-KO) mice. Our results revealed

that GSC005 tumors grew more rapidly and resulted in shorter survival in Prf1-KO mice compared to C57BL/6J mice (Supplementary Figure 2). However, staining for GFAP showed no significant difference in the infiltration of GFAP + cells into the tumors, suggesting that perforin may play a distinct role compared to IFN signaling in GBM progression and invasion (Supplemental Figure 2). Further studies using GBM patient-derived xenografts in immunodeficient mice, with or without reconstitution of human immune cells, would help to elucidate the role of immune factors in regulating tumor invasion.

CD73 is commonly expressed in malignant cells, where it promotes an immunosuppressive environment and contributes to therapy resistance. Recent studies have shown that CD73 is also expressed in tumor stromal cells,[37] such as cancer-associated fibroblasts, macrophages, and vesicles, where it facilitates tumor metastasis and therapy resistance. In our study, single-cell RNA sequencing of GBM patients revealed that CD73 is highly expressed in tumor-infiltrating brain cells, including oligodendrocytes, OPCs, astrocytes, and neurons. The expression of CD73 in these cells is influenced by host and therapeutic type-I and type-II IFN signaling, which modulates residual tumor invasion signaling. Our findings suggest that CD73 promotes GBM invasion in immunocompetent mice through intrinsic IFN responses. Targeting CD73 could enhance anti-tumor immune responses while simultaneously inhibiting tumor invasion. Furthermore, CD73 has been shown to regulate chemotherapy sensitivity[38,39] and tumor angiogenesis.[40] Several anti-CD73 monoclonal antibodies (e.g. BMS986179, TJ004309, IPH5301) and selective small molecule inhibitors (e.g. AB680, LY3475070) have been tested in preclinical studies involving various solid tumors.[41,42] However, additional research is needed to identify which CD73 monoclonal antibodies and small inhibitors are capable of crossing the BBB and enhancing their anti-tumor efficacy in the treatment of GBM.

In conclusion, this study explored the immune regulation of glioma invasion using murine GBM models in both immunodeficient and immunocompetent mice. We demonstrated that interferon signaling in both brain and tumor cells reciprocally modulates glioma invasion. These findings provide new insights into how immune signaling and brain cells contribute to GBM invasion, highlighting potential therapeutic targets for improving GBM treatment outcomes.

Study approval

Animal experiments were conducted under Augusta University IACUC protocol no. 2023-1098.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology Advances* (https://academic.oup.com/noa).

Keywords

CD73 | epithelial-mesenchymal-transition | glioblastoma | interferon

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Conflict of interest statement. None declared.

Authorship statement

B.H. designed experiments. K.K., J.Y., B.H., R.P., N.T., Y.O. and Q.W. performed the experiment. E.D. and R.P. performed bulk mRNA-seq and scRNA-seq analysis. B.H. wrote and revised the manuscript. B.K. and D.H.M. edited and reviewed the manuscript.

Data availability

The data used to support the findings of this study are available within this article.

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