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Posted Date: 4 November 2025

doi: 10.20944/preprints202511.0260.v1

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Article

Cancer Stem-Like Cells Orchestrate Perivascular Dissemination of Malignant Peripheral Nerve Sheath Tumors via Tumor-Associated Macrophages and Astrocytes

Short Title: CSLC–TAM–Astrocyte Axis Drives Perivascular MPNST Spread

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Highlights

- Nestin⁺/BuChE⁺ cancer stem-like cells exploit leptomeningeal and perivascular pathways to drive intracranial MPNST dissemination.
- Tumor-associated macrophages and reactive astrocytes establish a pro-invasive, immunosuppressive microenvironment that promotes tumor progression.
- ENU-induced rat model reproduces human MPNST brain infiltration patterns and provides a platform to target the CSLC–TAM–astrocyte axis

Abstract

Background: Malignant peripheral nerve sheath tumors (MPNSTs) of cranial nerves rarely invade brain parenchyma but frequently disseminate along perivascular spaces (PVS). Cancer stem-like cells (CSLCs) drive this process by contributing tumor spread and local microenvironmental disruption. This study investigated the distribution of nestin-positive CSLCs and associated glial-immune responses during PVS invasion in trigeminal MPNSTs. **Methods:** In an N-ethyl-N-nitrosourea (ENU)-induced rat model, trigeminal MPNSTs developed in 33% of exposed animals. Twenty-seven MRI-detected tumors were histologically analyzed. CSLCs were identified by nestin immunolabeling and butyrylcholinesterase (BuChE) histochemistry, while microvasculature, tumor-associated macrophages (TAMs), and reactive astrocytes were visualized using LEA-FITC, Iba-1, and GFAP immunostaining. **Results:** Tumors extended through leptomeningeal and Virchow–Robin spaces, accompanied by marked neuroinflammation, astrogliosis, and TAM recruitment (~15% of tumor mass). Nestin/BuChE-positive CSLCs clustered at PVS margins near the glia limitans, and subsets breached the astrocytic barrier, infiltrating adjacent parenchyma in close association with Iba-1/LEA-positive TAMs. **Conclusions:** CSLCs exploit PVS as invasive corridors in intracranial MPNSTs. TAMs and astrocytes establish a supportive microenvironment that promotes barrier disruption, immune evasion, and directional tumor spread. This ENU model recapitulates key features of CNS dissemination and provides a platform to explore the CSLC–TAM–astrocyte axis for targeted therapeutic strategies.

Keywords: cancer stem-like cells (CSLCs); malignant peripheral nerve sheath tumor (MPNST); N-ethyl N-nitrosourea (ENU); perivascular space (PVS); tumor-associated macrophages (TAMs)

1. Introduction

Malignant peripheral nerve sheath tumors (MPNSTs) are rare and highly aggressive sarcomas of Schwann cell lineage, accounting for 5–10% of all soft tissue sarcomas [1]. Their estimated incidence is 1.46 cases per million individuals, most frequently affecting young to middle-aged adults and strongly associated with neurofibromatosis type 1 (NF1) [2,3]. Despite advances in genetic and molecular profiling, MPNSTs remain therapeutically challenging, with limited response to surgery, radiotherapy, or chemotherapy. Infiltrative growth, perineural extension, and leptomeningeal dissemination contribute to the poor prognosis, and in intracranial or unresectable cases, median survival often falls below five months [4]. These clinical features underscore the urgent need to better understand the cellular and microanatomical mechanisms that support MPNST invasion.

One underexplored aspect of MPNST biology is the role of perivascular (Virchow–Robin) spaces (PVS). These fluid-filled extensions of the subarachnoid space accompany penetrating vessels into the brain parenchyma, forming dynamic conduits for cerebrospinal fluid (CSF), interstitial fluid exchange and immune surveillance [5]. Beyond their physiological roles in glymphatic clearance and neuroinflammation [6], PVS serve as permissive routes for tumor cell migration, with the structural continuity between leptomeninges, vasculature, and perivascular astrocytic endfeet creating specialized microenvironments that malignant cells can exploit.

The tumor microenvironment (TME) plays a pivotal role in MPNST progression. It includes Schwann cells, fibroblasts, endothelial and perineural cells, mast cells, extracellular matrix proteins, and a diverse immune infiltrate [7]. Among these, tumor-associated macrophages (TAMs) are predominant immune cell population, often polarizing toward immunosuppressive and pro-tumoral phenotypes that enhance invasion, angiogenesis, and metastasis [8,9]. In parallel a subset of tumor cells with stem-like properties, the so-called cancer stem-like cells (CSLCs), arise from neural crest-derived Schwann cell precursors, contribute to tumor heterogeneity, therapeutic resistance, and recurrence [10]. In MPNSTs, these CSLCs express canonical stemness-associated markers such as Prominin-1 (CD133), Octamer-binding transcription factor 4 (Oct4), and nestin [11,12].

Nestin, a class VI intermediate filament protein, plays a pivotal role in the biology of MPNSTs [13]. While normally expressed in neural progenitor cells, nestin is markedly upregulated in these tumors, paralleling its association with malignancy in other brain neoplasms such as gliomas and supporting its function as a marker of aggressive behavior [14]. This protein facilitates the establishment of the perivascular niche, promotes tumor cell proliferation, and enhances resistance to hypoxic stress through HIF-1 α -mediated signaling pathways [15–17]. Notably, nestin-positive cancer stem-like cells (nestin⁺ CSLCs) frequently cluster around blood vessels, suggesting that perivascular regions may serve as reservoirs of stemness and sites that foster invasive potential [18,19]. Complementing this observation, butyrylcholinesterase (BuChE), an enzyme associated with neurogenesis and oncogenic transformation [20–22], has also been implicated in tumor biology. In our previous work, BuChE expression was detected in perivascular tumor aggregates in a glioma rat model, suggesting its potential role as a histochemical marker of these niches [23].

Despite significant progress in defining the molecular landscape of MPNSTs, including mutations in NF1, TP53, and ERBB2 [2,3], the cellular pathways underpinning intracranial dissemination remain poorly understood [24]. Animal models provide essential platforms to dissect these mechanisms. Prenatal exposure to N-ethyl-N-nitrosourea (ENU) in rodents recapitulates human MPNST histopathology, genetic alterations, and invasive behavior, enabling systematic analysis of tumor–microenvironment interactions [25,26].

In this study, we investigate the spatial interplay between nestin⁺ CSLCs, TAMs, and reactive astrocytes in perivascular compartments of ENU-induced rat MPNST. Using histological, immunohistochemical, and lectin-based approaches, we demonstrate that CSLCs exploit PVS for

directional invasion, while TAMs and astrocytes define a permissive glial-immune microenvironment.

2. Materials and Methods

2.1. Animals

In accordance with the 3Rs principles (Replacement, Reduction, Refinement) [27], a subgroup of 74 rats from a previous study [28] was re-analyzed. In that study, six pregnant Sprague–Dawley rats received a single intraperitoneal injection of N-ethyl-N-nitrosourea (ENU; 80 mg/kg, Sigma-Aldrich, Ref. E2129) on gestational day 15. Their 74 offspring were raised for 6–9 months under standard housing conditions (22 ± 1 °C, $55 \pm 5\%$ humidity, 12:12 h light/dark cycle) with food and water provided ad libitum. All procedures complied with EU Directive 2010/63/EU and Spanish legislation (RD 53/2013) and were approved by the UPV/EHU Animal Ethics Committee (Ref. CEEA/82b/2010/LAFUENTESANCHEZ).

2.2. Tumor Screening and Tissue Processing

MPNSTs were identified by magnetic resonance imaging (MRI; 4.7 T BIOSPEC BMT 47/40, Bruker) at ICTS BioImagen Complutense (BioImac). Animals were anesthetized with isoflurane (4% for induction, 2% for maintenance) and administered gadolinium-DTPA (1.5 mL/kg, i.v.) prior to acquisition of T1-weighted coronal and sagittal sequences (TR/TE = 700/15 ms). Image analysis was performed using Fiji software (ImageJ v2.14.0, NIH).

After tumor detection, rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains and trigeminal nerves were extracted and post-fixed overnight in the same fixative, then either paraffin-embedded or cryoprotected in 30% sucrose. Coronal sections (4 μ m for paraffin, 60 μ m for cryosections) were obtained using a Leica HM325 rotary microtome and an HM430 freezing microtome, respectively.

2.3. Histology and Immunohistochemistry

Hematoxylin and eosin (H&E) staining was performed for histopathological evaluation of MPNSTs, including spindle cell morphology, mitotic figures, necrosis, hemorrhage, and microvascular proliferation.

Immunophenotypic characterization was conducted using the avidin–biotin complex method (Elite Vectastain ABC Kit, Vector Laboratories) with antibodies against Ki-67, EMA, SYP, S-100, NF, EGFR, and TP53 (Table 1). Tumor-associated macrophages (TAMs), astrocytes, and cancer stem-like cells (CSLCs) were labeled with Iba-1, GFAP, and nestin antibodies. Paraffin sections (4 μ m) were deparaffinized, rehydrated, and subjected to antigen retrieval (citrate or trypsin), followed by incubation with primary antibodies, biotinylated secondary antibodies, and ABC-peroxidase. Immunoreactivity was visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. Negative controls omitted the primary antibodies.

Double immunofluorescence was performed to analyze perivascular dissemination and associated cellular components. Microvasculature was visualized using *Lycopersicon esculentum* lectin conjugated to FITC (LEA-FITC; 1:100, Sigma-Aldrich). Sections were incubated overnight at 4 °C with a cocktail of primary antibodies (Iba-1, GFAP, and nestin), followed by Alexa Fluor–conjugated secondary antibodies (Alexa 488, 568, and 647; 1:400, Invitrogen). Nuclei were counterstained with Hoechst 33258. Images were acquired using a Zeiss LSM800 confocal microscope (SGIker Microscopy Facility, UPV/EHU, Leioa, Spain) and analyzed with ImageJ v2.14.0 (NIH) using standardized calibration and processing workflows.

Table 1. Primary and secondary antibodies used for immunohistochemistry (IHC) and immunofluorescence assay (IF).

Antibodies	Target protein	Dilution / Clone	Catalogue No.	Supplier
Primary antibodies				
Nestin	Neuroepithelial stem cell protein	1:75, mAb (mouse)	sc-33677	Santa Cruz Biotechnology
GFAP	Glial fibrillary acidic protein	1:200, pAb (rabbit)	Z0334	Dako
GFAP	Glial fibrillary acidic protein	1:200, pAb (goat)	Ab7260	Abcam
Ki-67 (MIB-5)	Nuclear proliferation marker	1:75, mAb (mouse)	M7248	Agilent Technologies
Iba-1	Ionized calcium-binding adaptor protein-1	1:500, pAb (rabbit)	019-19741	Fujifilm Wako
EMA	Epithelial membrane antigen	1:100, pAb (rabbit)	ab19898	Abcam
SYP	Synaptophysin	1:75, mAb (mouse)	IR776	Dako
S100	Schwann cell marker	1:100, mAb (mouse)	MA1-26621	Thermo Fisher Scientific
NF	Neurofilament protein	1:100, mAb (mouse)	AB1991	Chemicon International
EGFR	Epidermal growth factor receptor	1:150, mAb (mouse)	M7239	Dako
TP53	Tumor suppressor p53	1:75, mAb (mouse)	pAb240	Dako
Secondary antibodies				
Alexa Fluor 647	Donkey anti-rabbit IgG	1:200	A31573	Invitrogen
Alexa Fluor 488	Donkey anti-mouse IgG	1:200	A21202	Invitrogen
Alexa Fluor 568	Donkey anti-goat IgG	1:200	A11057	Invitrogen

Abbreviations: mAb, monoclonal antibody; pAb, polyclonal antibody; GFAP, glial fibrillary acidic protein; Ki-67, nuclear proliferation marker; Iba-1, microglia/macrophage-specific calcium-binding protein; EMA, epithelial membrane antigen; SYP, synaptophysin; S100, Schwann cell marker; NF, neurofilament protein; EGFR, epidermal growth factor receptor; TP53, tumor suppressor protein p53.

2.4. Histochemistry

Free-floating coronal sections (60 μm) were processed for butyrylcholinesterase (BuChE) histochemistry as previously described [29]. Briefly, sections were preincubated for 20 min with BW284C51 (0.05 M) to inhibit acetylcholinesterase activity and incubated overnight at room temperature in a reaction mixture containing butyrylthiocholine iodide (1 mg/mL), sodium citrate (5%, 0.1 M), copper sulfate (10%, 30 mM), BW284C51 (10%, 0.05 mM), potassium ferricyanide (10%, 5 mM), and Tris-maleate buffer (65%, 0.1 M, pH 6.0). The following day, sections were rinsed, mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene, and coverslipped with mounting medium.

2.5. Labelling Index Quantification

In 25 representative MPNST samples (see supplementary Table S1), tumor proliferative activity and TAM density were quantified using Ki-67 and Iba-1 immunolabeling indices (LI). Positive cells were counted as a percentage of 400 tumor cells within the areas showing the highest

immunoreactivity, using a field size of 62,500 μm^2 at $\times 400$ magnification. Only isolated immune-positive cells not associated with vascular structures were included. All quantifications were performed by a single blind observer to minimize variability.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS Statistics 29.0 (IBM, Spain). Normality and variance homogeneity were assessed with the Kolmogorov–Smirnov and Levene’s tests. Group differences were evaluated using one-way ANOVA with appropriate post hoc tests, and Student’s *t*-test for pairwise comparisons. Correlations between tumor proliferation and TAM density were examined using Pearson’s correlation coefficient. Data are presented as mean $\pm$ SD, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Imaging and Histopathological Features of Basal and Convexity ENU-Induced MPNSTs

Among 74 ENU-exposed rats (36 females, 38 males), 33.7% developed MPNSTs, with a slight male predominance (M: F 1.3:1) and peak incidence at 6–7 months (Table 2). MRI revealed enhancing tumors arising from the trigeminal (Gasserian) nerve root, extending along the meningeal sheath and disseminating through subarachnoid and perivascular spaces toward cortical convexities, interhemispheric fissure, and penetrating vessels, causing cortical compression and midline shift (Figure 1A,B).

Table 2. Chronology of MPNST detection in Sprague Dawley rats after ENU prenatal exposure.

Age (months)	Total Rats (M/F)	MPNST Cases (N)	MPNST %	MPNST Cases (M/F)	MPNST % (M/F)
6	29 (14/15)	12	41.37	7/5	50/33.3
7	15(6/9)	7	46.66	3/4	50/44.4
8	12(8/4)	4	33.3	3/1	37.5/25
9	18(8/10)	2	11.11	1/1	12.5/10
Total	74(36/38)	25	33.7	14/11	38/28.9

MPNST % indicates the proportion of MPNST cases among rats of that age. MPNST % (M/F) indicates the proportion of male or female MPNST cases relative to the total male or female MPNST cases. **Abbreviations:** M, male; F, female.

Histologically, 17 of 25 analyzed tumors showed basal brain dissemination, characterized by high cellularity, marked pleomorphism, frequent mitoses, hemorrhage and occasionally entrapping Schwann cells (Figures 1C and 2A,B). Basal tumors exhibited focal S-100 expression (Figures 1E and 2C), strong synaptophysin (Figure 2D), moderate Ki-67 labeling (Figure 2E), and scattered p53-positive nuclei (Figure 2F).

In contrast, eight convexity tumors extended into the interhemispheric fissure and Virchow–Robin spaces, displaying lower cellularity, spindle-shape morphology, rare mitoses, nuclear palisading, and dilated vessels (Figures 1D and 2G,L), with diffuse S100 staining (Figures 1F and 2J). moderate Ki-67 labeling (Figure 2K), and low p53 positivity (Figure 2L). Nestin was consistently expressed in both regions (Figure 1G,H), whereas GFAP, NF, EMA, EGFR, and NSE were negative. Overall proliferative activity, assessed by Ki-67, was $16.3\% \pm 8.6\%$, higher in basal tumors ($18.5\% \pm 8.9\%$) than in convexity lesions ($13.2\% \pm 7.02\%$) but not statistically significant ($p < 0.05$).

Clinically, tumor growth resulted in obstructive hydrocephalus, brainstem and cranial nerve involvement, and extensive leptomeningeal spread, contributing to elevated intracranial pressure and global brain distortion.

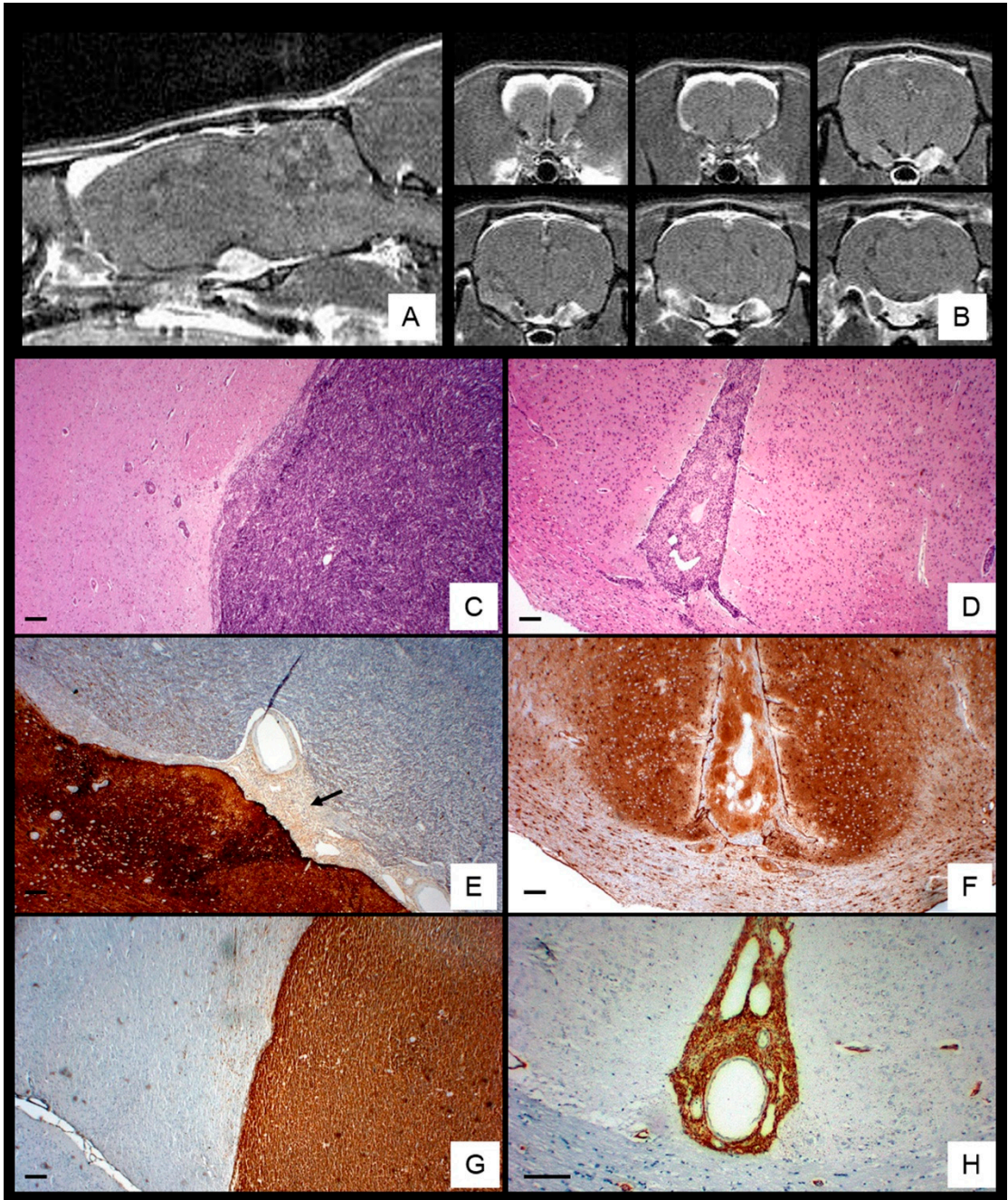


Figure 1. Anatomical and Histological Characterization of ENU-Induced MPNSTs. T1-weighted MRI showing sagittal (A) and coronal (B) sections of a rat head with an ENU-induced MPNST extending from the Gasserian ganglion sheath toward the cortical convexity, producing cortical compression and midline shift. H&E staining reveals tumor spreading at the brain base (C) and within the interhemispheric subarachnoid space (D). S-100 immunolabeling shows focal positivity near the parenchyma (black arrow) (E) and diffuse strong expression at the cortical convexity (F). Nestin immunoreactivity intense in both regions of dissemination sites (G,H). Scale bar: 100 μ m.

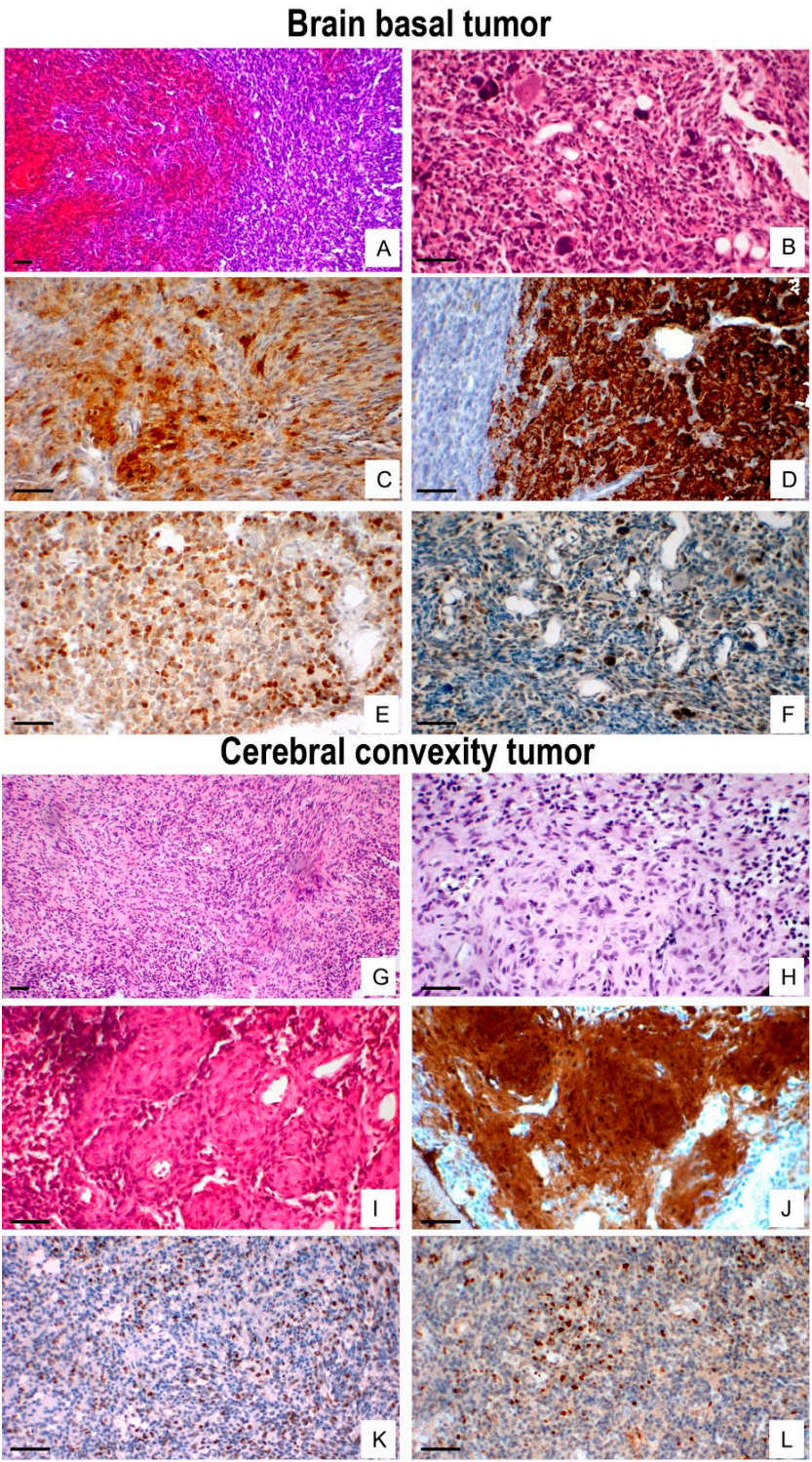


Figure 2. Immunophenotypic profile of ENU-induced MPNST. Basal tumors arising from the Gasserian ganglion sheath exhibit hemorrhage, high cellularity (A), marked nuclear atypia, and entrapped Schwann cells (B). Immunohistochemistry shows focal S-100 expression (C), strong synaptophysin immunoreactivity (D), moderate Ki-67 labelling (E), and scattered p53-positive nuclei (F). Convexity tumors display spindle-shaped, hyperchromatic cells (G), compact fascicular architecture (H), and hyalinized vessels (I), with diffuse S-100 (J), moderate Ki-67 (K), and low p53 expression (L). Scale bar: 50 μ m.

3.2. BuChE Expression Highlights Perivascular and Leptomeningeal Invasion

In ENU-induced MPNSTs, BuChE histochemistry revealed markedly elevated enzymatic activity, highlighting regions enriched in nestin⁺ cancer stem-like cells (CSLCs) compared to adjacent

brain parenchyma. Coronal sections demonstrated extensive infiltration of BuChE⁺ tumor cells originating at the brain base and advancing along the subarachnoid space and meningeal sheath toward the interhemispheric fissure, with contralateral dissemination evident (Figure 3A). At higher magnification, dense clusters of BuChE⁺ cells occupied perivascular spaces surrounding penetrating cortical vessels (Figure 3B), while fibrillar aggregates in leptomeningeal compartments formed elongated, finger-like projections consistent with directional, stem cell-driven invasion (Figure 3C). These observations indicate that CSLCs actively mediate intracranial perivascular and leptomeningeal dissemination, exploiting anatomical corridors as invasive niches.

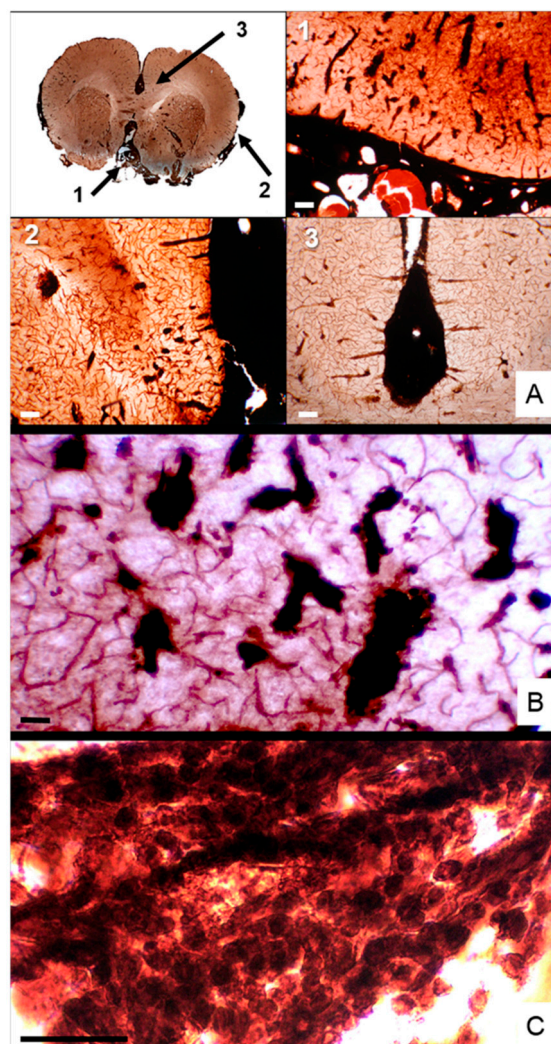


Figure 3. Leptomeningeal dissemination showed by Butyrylcholinesterase staining. A coronal rat brain section showing intense BuChE staining delineating ENU-MPNST spread through interconnected subarachnoid and perivascular spaces (A). Tumor dissemination extends from the basal leptomeninges (1), laterally along the meningeal sheath (2), and reaches the interhemispheric fissure (3). (B) Tumor dissemination toward the parenchyma occurs via perivascular spaces surrounding penetrating vessels. (C) Fibrillar BuChE⁺ cell clusters are seen in leptomeningeal compartments. Scale bar: 50 μm.

3.3. Perivascular Infiltration of Nestin⁺ Cancer Stem-Like Cells and Glial-Immune Interactions

Iba-1 immunohistochemistry revealed abundant tumor-associated macrophages (TAMs) within the subarachnoid space of ENU-induced MPNSTs, with a mean labeling index of $14.4 \pm 5.8\%$. TAM density was higher in basal tumors ($16.5 \pm 5.8\%$) than in cortical lesions ($11.0 \pm 4.4\%$), although this difference was not statistically significant ($p > 0.05$). TAM density positively correlated with proliferative activity (Ki-67 labeling index; $r = 0.574$, $p = 0.003$, Pearson's correlation), suggesting a

functional association with tumor growth (Figure S1). Morphological analysis revealed location-specific heterogeneity: amoeboid TAMs predominated within tumor cores, intermediate forms were observed along arachnoid and pia mater, and ramified microglia indicative of a resting state were present in adjacent non-tumoral parenchyma (Figure 4). This functional specialization emphasizes the role of TAMs in modulating CSLC-mediated invasion within perivascular and leptomeningeal niches.

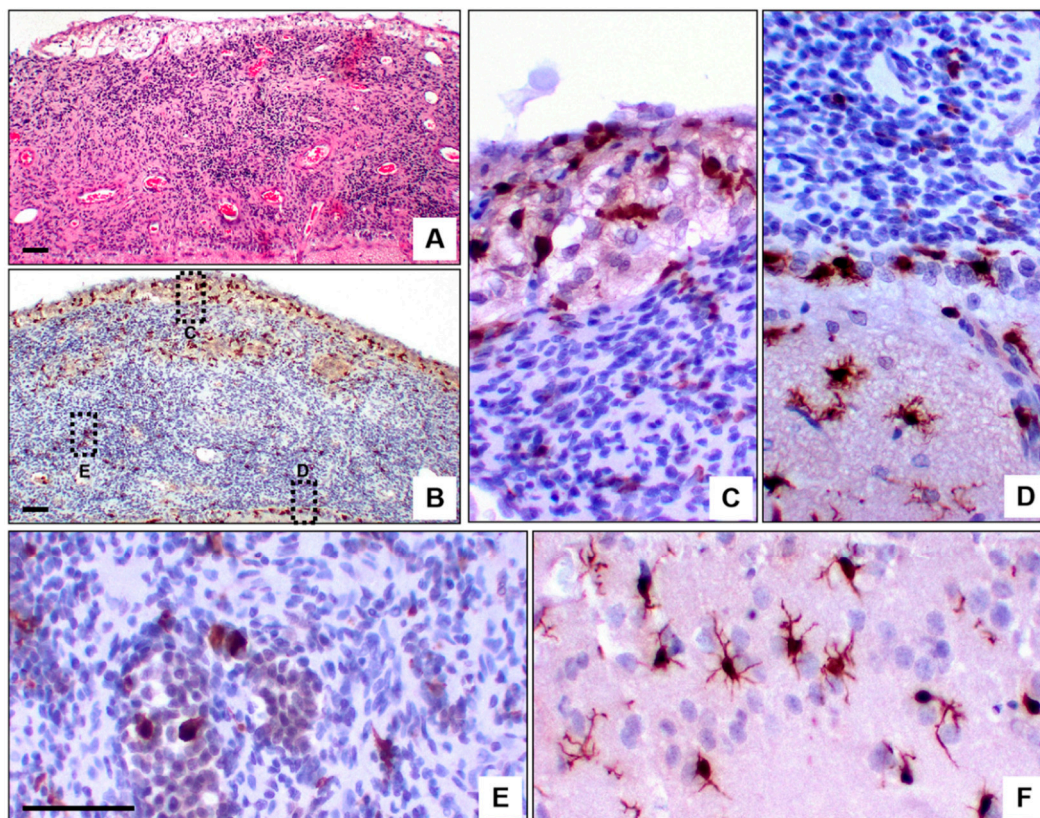


Figure 4. Morphological heterogeneity of tumor-associated macrophages (TAMs). H&E staining of an MPNST with leptomeningeal dissemination (A) shows abundant Iba-1⁺ TAMs. (B) Distinct TAM morphologies are observed depending on location (highlighted in rectangles): intermediate forms within the arachnoid (C) and pia mater (D), amoeboid forms in the tumor core (E), and ramified microglia reflecting a resting state in adjacent non-tumoral parenchyma (F). Scale bar: 50 μ m.

Confocal microscopy further demonstrated that nestin⁺ CSLCs accumulated along vascular walls and formed perivascular aggregates, which were closely bordered by GFAP⁺ reactive astrocytes at the glia limitans and enriched with short-ramified Iba-1⁺ TAMs intermingled with hyperreactive astrocytic processes (Figures 5 and 6A,B). In deeper cortical regions, CSLCs concentrated at perivascular invasive fronts, creating a coordinated glial-immune interface that delineated the advancing tumor edge. Individual nestin⁺ CSLCs occasionally breached the astrocytic barrier, infiltrating adjacent parenchyma while remaining partially enclosed within a dual protective layer composed of inner GFAP⁺ astrocytes and outer Iba-1⁺ TAMs (Figure 6C,D).

Collectively, these findings highlight that CSLCs mediate intracranial perivascular and leptomeningeal dissemination, orchestrating a dynamic interplay with TAMs and reactive astrocytes that promotes directional invasion, local immune modulation, and niche conditioning. This CSLC-TAM-astrocyte crosstalk establishes a permissive microenvironment that mirrors pre-metastatic niches described in other CNS malignancies and underscores the potential of targeting these cellular interactions to limit MPNST progression.

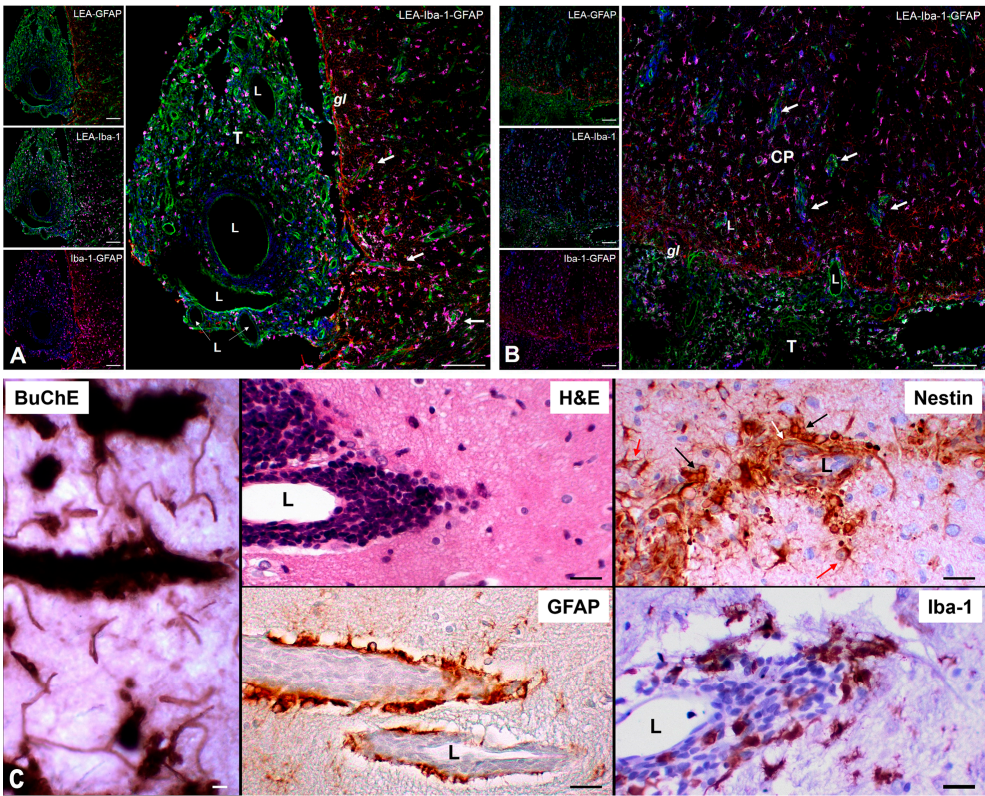


Figure 5. Perivascular dissemination of ENU-induced MPNSTs. (A,B) Confocal immunofluorescence showing penetrating microvessels (white arrows) labeled with LEA-FITC (green and active glial reaction (GFAP, red) with Iba-1⁺ TAMs (purple) at the tumor–brain interface; nuclei counterstained with Hoechst (blue). (C) Perivascular space (PVS) histology demonstrates strong BuChE immunoreactivity at the invasion front. nestin⁺ CSLCs display diverse morphologies: small round clusters (black arrow), elongated perivascular cells (white arrow), and astrocyte-like cells at the tumor margin (red arrow). The spatial arrangement emphasizes the interplay between CSLCs, TAMs, and astrocytes within invasion-permissive niches. Scale bars: A–B, 100 μ m; C, 50 μ m. Abbreviations: CP, cerebral parenchyma; T, tumor; L, vessel lumen; gl, glia limitans.

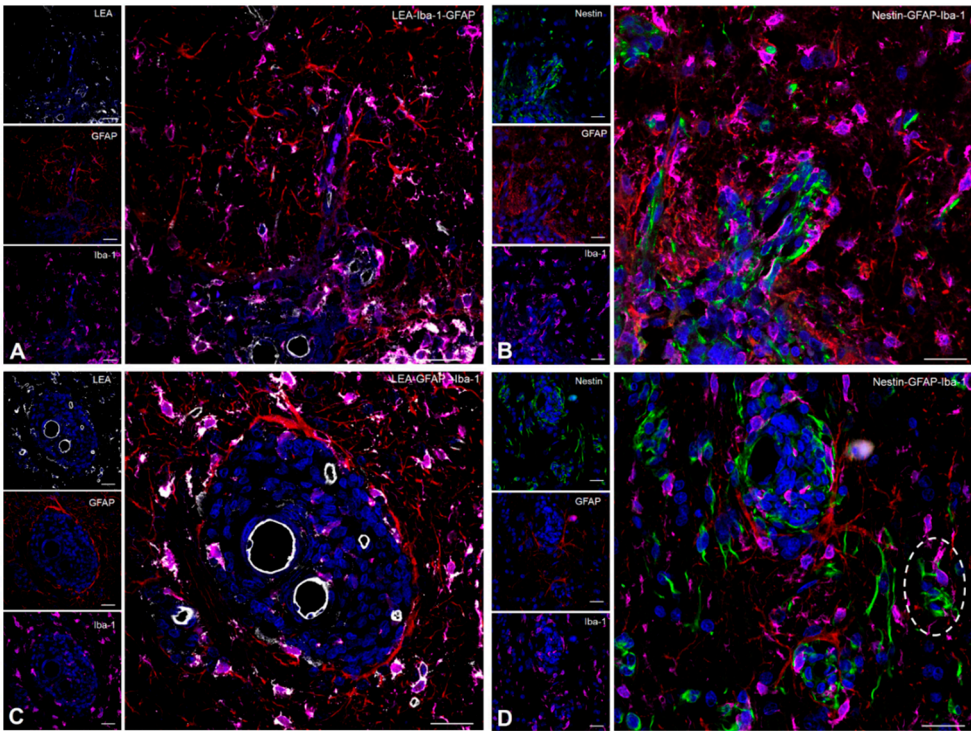


Figure 6. CSLC–Glial–Immune Interactions at the Invasive Front of MPNSTs. Confocal immunofluorescence images illustrate the coordinated interplay between nestin⁺ CSLCs (green), GFAP⁺ reactive astrocytes (red), and Iba-1⁺ tumor-associated macrophages (TAMs, purple) during tumor progression. **(A,B)** At the primary tumor margin, abundant penetrating microvessels (LEA, white) are surrounded by a dense network of reactive astrocytes and ramified TAMs, with Nestin⁺ CSLCs aligned along vascular walls and within perivascular niches. **(C)** In deeper cortical regions, CSLCs accumulate at the perivascular invasive front, interacting with GFAP⁺ astrocytic processes and TAMs to breach the glial barrier. **(D)** Individual Nestin⁺ tumor cells infiltrate the adjacent parenchyma, enclosed by a dual protective glial–immune barrier: an inner layer of GFAP⁺ astrocytes and an outer ring of Iba-1⁺ TAMs. Scale bar: 20 μ m.

4. Discussion

In ENU-induced rat model of intracranial malignant peripheral nerve sheath tumors (MPNSTs), perivascular (Virchow–Robin) and leptomeningeal compartments emerge as preferential routes for tumor dissemination from the trigeminal root into the brain. These anatomical corridors represent permissive niches enriched in nestin⁺ cancer stem-like cells (CSLCs) and Iba-1⁺ tumor-associated macrophages (TAMs), forming a coordinated microenvironment that promotes invasion and tumor progression.

Tumor proliferation was significantly higher at the primary trigeminal root site compared with distant cortical lesions, underscoring the influence of local tissue architecture and microenvironmental cues on growth dynamics. Histologically, neoplastic cells infiltrated Virchow–Robin spaces and subarachnoid routes, occasionally breaching the glia limitans to colonize adjacent parenchyma, a pattern reminiscent of leptomeningeal dissemination observed in high-grade gliomas, ependymomas, and metastatic carcinomas [30,31]. Impaired cerebrospinal fluid dynamics within these compartments may exacerbate interstitial fluid accumulation, neuroinflammation, peritumoral edema, and immune evasion, suggesting that glymphatic dysfunction contributes to tumor spread [32–36].

A distinctive feature of this model is the consistent presence of nestin⁺ CSLCs within perivascular and leptomeningeal niches, often arranged in chain-like patterns suggestive of collective migration. Their close association with vascular structures underscores the relevance of endothelial–stem cell interactions in maintaining stemness and directing invasion [37,38].

The interplay between immune and stem-like components appears central to this process. TAMs were abundant across primary and disseminated ENU-MPNST sites, displaying marked morphological heterogeneity, from amoeboid forms in tumor cores to transitional phenotypes at invasion fronts. TAM density correlated positively with the Ki-67 proliferation index, suggesting an active role in promoting tumor expansion. Dissemination along perivascular routes was also associated with overexpression of butyrylcholinesterase (BuChE). Although BuChE has been implicated in tumorigenesis in several malignancies [21,22,39,40], its role in MPNSTs remains largely unexplored. The present findings indicate that BuChE may enhance CSLC motility and modulate perivascular niche dynamics through TAM–astrocyte activation. In other malignancies, such as gliomas and non-small cell lung carcinoma, dysregulation in cholinergic metabolism polarizes TAMs toward immunosuppressive and proangiogenic phenotypes [41,42]. Consistently, Wang et al. (2025) demonstrated that BuChE regulates neuroinflammation and tumor immunosuppression via astrocyte and microglial activation [41].

At invasion fronts, nestin⁺ CSLC clusters were frequently surrounded by reactive astrocytes and TAMs, resembling the pre-metastatic niches described in gliomas and brain metastases [43]. These reactive glial and immune cells contribute to glia limitans disruption and extracellular matrix remodeling through cytokines (CCL2, IL-6, CSF1), metalloproteases (MMP2, MMP7, MMP9), and growth factors (VEGF, PDGF, EGF) [44]. Following disruption of the glia limitans, nestin⁺ CSLCs infiltrate the parenchyma and acquire invasive capacity, while PD-L1 expression facilitates local immune evasion [45,46]. Transcriptomic analyses of human MPNSTs support these findings, showing activation of STAT3, Sonic Hedgehog (SHH), and Wnt/ β -catenin pathways [47].

Together, these results support a model in which perivascular and leptomeningeal compartments act as permissive microenvironments for MPNST dissemination, where nestin⁺/BuChE⁺ CSLCs, TAMs, and reactive astrocytes interact to create a pro-invasive, immunomodulatory niche. The morphological heterogeneity of TAMs likely reflects functional specialization, with TAM-derived exosomes, cytokines, and metabolic cues likely orchestrating CSLC seeding and expansion [48]. As summarized in Figure 7, we propose a mechanistic framework for the CSLCs-TAM-astrocyte axis driving intracranial dissemination in ENU-induced MPNSTs

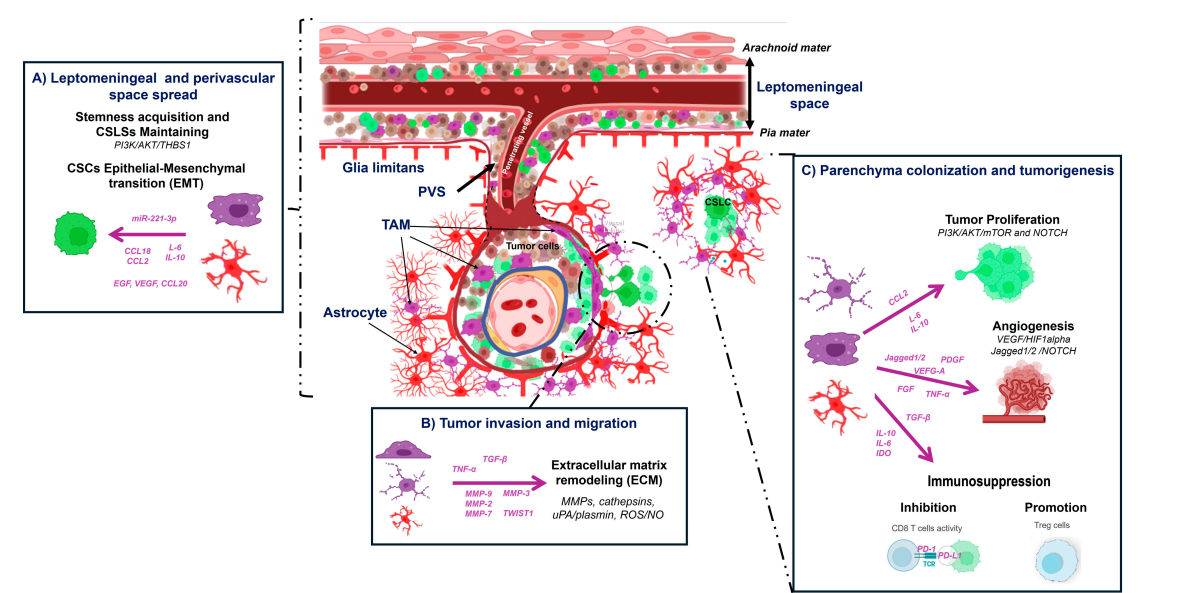


Figure 7. Proposed mechanisms of intracranial dissemination in ENU-induced MPNSTs. Tumor-associated macrophages (TAMs) and astrocytes support cancer stem-like cells (CSLCs) and drive ENU-MPNST dissemination via three interconnected mechanisms: **(A)** Leptomeningeal and perivascular spread. Enhancement of CSLC stemness and mesenchymal plasticity through cytokine secretion (CCL2, IL-6, IL-10), activation of PI3K/AKT and NOTCH pathways, release of growth factors (EGF, FGF), and exosomal delivery of miR-221-3p. **(B)** Tumor invasion and migration. Remodeling of the extracellular matrix (ECM) via MMPs, cathepsins, uPA/plasmin, ROS/NO, and exosomal proteases, promotes epithelial-to-mesenchymal transition (EMT) through EGF, VEGF, and CCL20 signaling. **(C)** Parenchyma colonization and tumorigenesis. Tumor proliferation is enhanced through PI3K/AKT/mTOR and NOTCH pathways activation, while angiogenesis is induced via VEGF/HIF1α and Jagged1/2–NOTCH signaling. The release of VEGF, PDGF, TGF-β, FGF, TNF-α, and MMPs sustains vascular remodeling and metastatic growth. In parallel, secretion of immunosuppressive mediators (IDO, IL-10, IL-6, TGF-β) inhibits CD8⁺ T-cell activity and promotes Treg expansion, generating an immunosuppressive tumor microenvironment. **Abbreviations:** TAMs, tumor-associated macrophages; CSLCs, cancer stem-like cells; ENU-MPNST, N-ethyl-N-nitrosourea-induced malignant peripheral nerve sheath tumor; PVS, perivascular space; CCL2, C-C motif chemokine ligand 2; IL, interleukin; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; EGF, epidermal growth factor; FGF, fibroblast growth factor; miR-221-3p, microRNA-221-3p; ECM, extracellular matrix; MMPs, matrix metalloproteinases; uPA, urokinase-type plasminogen activator; ROS, reactive oxygen species; NO, nitric oxide; VEGF, vascular endothelial growth factor; HIF1α, hypoxia-inducible factor-1α; EMT, epithelial-to-mesenchymal transition; CCL20, C-C motif chemokine ligand 20; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IDO, indoleamine 2,3-dioxygenase; Tregs, regulatory T cells. Adapted from Verona et al. [44] and Basak et al. [46]. Created with BioRender.com.

Limitations

Limitations of this study include: (i) absence of functional assays directly testing CSLC behavior; (ii) TAM characterization limited to Iba1 without polarization markers (e.g., CD206, iNOS) [49]; (iii)

indirect inference of glymphatic involvement; and (iv) the unvalidated role of BuChE in human MPNSTs. Despite these constraints, the ENU model faithfully recapitulates key features of intracranial MPNST invasion and provides a robust platform for investigating tumor–microenvironment interactions.

5. Conclusions

Leptomeningeal and perivascular spaces represent critical anatomical routes for intracranial MPNST dissemination. The coordinated interplay among nestin⁺/BuChE⁺ CSLCs, TAMs, and reactive astrocytes establishes a permissive niche that promotes invasion and immune evasion. These findings provide a mechanistic framework for developing targeted therapeutic strategies aimed at disrupting the CSLC–TAM–astrocyte axis to limit tumor progression and improve outcomes in this aggressive disease.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Graphical representation of Ki-67 and Iba-1 labeling indices (LI) according to the anatomical region of MPNST dissemination; Table S1: Dataset of 25 ENU-induced MPNST samples selected for quantitative analysis.

Author Contributions: Conceptualization, S.B. and J.V.L.; Methodology, S.B. and N.O.; Software, S.B. and L.Z.; Validation, S.B., L.Z., and J.V.L.; Formal analysis, A.C. and J.V.L.; Investigation, S.B.; Resources, S.B.; Data curation, S.B.; Writing—original draft, S.B.; Writing—review and editing, A.C. and J.V.L.; Supervision, A.C. and A.M.; Project administration, A.M.; Funding acquisition, A.M. All authors have read and approved the final manuscript.

Funding: This research was funded by the Spanish Ministry of Science, Innovation and Universities (PID2021-126434OB-I00) and Basque Government (IT1706-22, 2022333034, 2023333012 and 2024333005) and Recovery and Resilience Facility—NextGenerationEU (INVESTIGO program).

Institutional Review Board Statement: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee for Animal Research of the University of the Basque Country (Ref. CEEA/82b/2010/LAFUENTESANCHEZ).

Data Availability Statement: The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors thank Encarnación Fernández-Valle and David Castejón Ferrer from ICTS Bioimagen Complutense (BIOIMAC) for their support with MRI analysis, and Aitor Jauregi for technical assistance with immunohistochemical studies. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

BuChE	Butyrylcholinesterase
CNS	Central nervous system
CFS	Cerebrospinal fluid
CSLC	Cancer stem-like cell
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
ENU	N-ethyl-N-nitrosourea
GFAP	Glial fibrillary acidic protein
Iba-1	Ionized calcium-binding adaptor protein-

Ki-67	Nuclear proliferation marker
LEA	<i>Lycopersicon esculentum</i> lectin
LI	Labelling index
MMPs	Matrix metalloproteinases
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic Resonance Imaging
NF	Neurofilament protein
NF1	Neurofibromatosis type 1
NSE	Neuron-specific enolase
PVS	Perivascular (Virchow-Robin) space
SYP	Synaptophysin
S100	Small acidic EF-hand calcium-binding proteins 100
TAM	Tumor-associated macrophage
TME	Tumor microenvironment
TP53	Tumor suppressor p53

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