

Original Articles

Galectin-3 depletion tames pro-tumoural microglia and restrains cancer cells growth

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ABSTRACT

Galectin-3 (Gal-3) is a multifunctional protein that plays a pivotal role in the initiation and progression of various central nervous system diseases, including cancer. Although the involvement of Gal-3 in tumour progression, resistance to treatment and immunosuppression has long been studied in different cancer types, mainly outside the central nervous system, its elevated expression in myeloid and glial cells underscores its profound impact on the brain's immune response. In this context, microglia and infiltrating macrophages, the predominant non-cancerous cells within the tumour microenvironment, play critical roles in establishing an immunosuppressive milieu in diverse brain tumours. Through the utilisation of primary cell cultures and immortalised microglial cell lines, we have elucidated the central role of Gal-3 in promoting cancer cell migration, invasion, and an immunosuppressive microglial phenotypic activation. Furthermore, employing two distinct *in vivo* models encompassing primary (glioblastoma) and secondary brain tumours (breast cancer brain metastasis), our histological and transcriptomic analysis show that Gal-3 depletion triggers a robust pro-inflammatory response within the tumour microenvironment, notably based on interferon-related pathways. Interestingly, this response is prominently observed in tumour-associated microglia and macrophages (TAMs), resulting in the suppression of cancer cells growth.

1. Introduction

The tumour microenvironment (TME) offers a rich spatiotemporal dynamic of cellular components. Apart from malignant cancer cells, there are a complex myriad of tissue resident and infiltrated peripheral immune system cells. This evolving mosaic of multifunctional cells makes the TME a rather complicated topic of study in the field, with great importance to develop novel therapies that may effectively be used in the most challenging cancers to treat.

Breast cancer has been recently placed as the most diagnosed cancer worldwide, with great affinity to colonise distant organs, specially the brain [1]. Thus, depending on tumour subtype, breast cancer brain metastases (BCBM) are very common and represent a major health issue with meagre survival rates amongst patients (ca. 12 months) [1]. In turn,

one of the most common forms of primary brain tumours is glioblastoma (GB), the deadliest types of brain cancer, where patients who undergo treatment have an average survival measured just in months (ca. 15 months) [2]. Despite primary brain tumours and the metastatic colonisation of the brain are completely different diseases, they grow under the influence of the unique immune microenvironment found in the central nervous system, which clearly drives the disease progression [1, 3].

BCBM and GB are considered immunologically “cold”, owing to their lack of tumour antigens, inefficient T-cell infiltration and an abundant presence of myeloid-derived immune cells [4]. Amongst the broad plethora of cells that constitute the TME, the most abundant non-cancerous cell type are microglia and infiltrated macrophages. Tumour-associated microglia and macrophages (TAMs) have been

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described to develop an anti-inflammatory phenotype and ultimately support BCBM and GB growth [5,6].

In that sense, one key component of the immunosuppressive behaviour of TAMs is Galectin-3 (Gal-3) [7,8]. This β -galactoside-binding protein is long known to be a poor prognostic marker in breast cancer [9] and glioblastoma [10]. Given the immunomodulatory role of Gal-3, a potential role of this lectin in driving a pro-tumoural TME in brain tumours is worth being considered. Previous works have shown how activated microglia are the major source of Gal-3 within the brain [7,11], together with astrocytes [12]. Therefore, this study has focused on the role of TAMs-associated Gal-3, in driving immunosuppression in BCBM and GB.

Our results describe a strong presence of Gal-3 closely associated to TAMs in BCBM and GB human resections, and *in vivo* models. Blocking Gal-3 *in vitro*, we managed to shape microglia activation, suppressing their anti-inflammatory phenotype and inhibiting cancer cells migration and invasion. Our *in vivo* models provided compelling evidence supporting a critical role of Gal-3 in driving TAMs-associated immunosuppression within BCBM and GB microenvironment. Transcriptomic analysis showed a strong activation of key innate and adaptive immune response pathways, thus, tempering pro-tumoural TAMs phenotype towards a more pro-inflammatory state, contributing to cytotoxic T-cell infiltration and tumour size reduction. Lastly, GB-bearing Gal-3 KO mice who underwent the standard chemotherapeutic agent, Temozolomide (TMZ), showed a synergistic effect in arresting tumour growth [13].

2. Material and methods

2.1. Cell culture and transfection

GL261 (mouse glioblastoma), BV2 (mouse microglia) and EO771 (mouse mammary carcinoma) cell lines were purchased from the American Type Culture Collection (ATCC, USA). For detailed culture conditions see Supp. data. BV-2 cells Gal-3 knockout were kindly provided by Prof Tomas Deierborg. For primary microglia culture, migration and invasion, please read Supplementary data.

2.2. RNA extraction and quantitative PCR

RNA was extracted from the cell lines using the RNeasy Kit (Qiagen, Netherlands). For protocol and primers information see Supp. data.

2.3. Western blotting assay

Membranes were incubated with anti-Gal 3 antibody (1:1000). GAPDH antibody (Sigma-Aldrich; 1:2000; USA) was used as a loading control. For protocol information see Supp. data.

2.4. Animals and surgery

Experiments were performed in 12-week-old mice C57BL/6 (wild-type, wt, n = 8) and Galectin-3 null mutant mice (Gal-3 KO, n = 8) with the same background, both lines obtained from Charles Rivers. Female mice were used for the BCBM model [14] and male mice for the GB model [15]. Animal experimentation was carried out in accordance with the European Community Council Directives (86/609/EU) and Spanish law (R.D. 53/2013 BOE 34/11370-420, 2013) for the use and care of laboratory animals. For *in vivo* protocol details, please see Supplementary data.

2.5. Immunohistochemistry and microscopy

Staining protocol was performed as previously described [15]. For full information, please see Supp. data.

2.6. Quantitation of tumour burden

All data were analysed blinded to experimental conditions. Tumour growth was histologically assessed as previously described [16,17], (Supp. Figure 2). For full information see Supp. data.

2.7. TAMs analysis

Colocalisation studies were analysed using the ImageJ software package (NIH) as previously described [18]. Full information on the plugins and software can be found in the Supp. Data.

2.8. Microarray analysis

To study the differences in genes expression between wt (n = 3) and Gal-3 KO mice (n = 3), Transcriptome Analysis Console (TAC) Software was used. Gene Set Enrichment analysis (GSEA) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) were also used to further analyse gene clusters comparisons and key biological pathways. For full information see Supp. data.

2.9. Human data and samples from tumour Bank collection

Samples and data were provided by Biobank HUB-ICO-IDIBELL, integrated in the Spanish Biobank Network and funded by Instituto de Salud Carlos III (PT20/00171) and by Xarxa de Bancs de Tumors de Catalunya (XBTC, Pla Director d'Oncologia de Catalunya). We downloaded the Cancer Genome Atlas (TCGA) data for the breast cancer (TCGA-BRCA) and the lower-grade glioma and glioblastoma (GBMLGG) database from UCSC Xena (<https://xena.ucsc.edu/>).

2.10. Statistical analysis

All data are presented as mean \pm SD. Unless otherwise stated, data were analysed using GraphPad Prism (v8.4). For 2 groups, Student's t-test (2-tailed) was used. For more than 2 groups, 1-way ANOVA with Tukey's multiple comparison test or two-way Anova with Bonferroni test were used and adjusted P values are reported. Statistical significance was defined as p < 0.05.

2.11. Data availability statement

Transcriptomic data were generated at the Genome and Sequencing facilities in the Institute of Biomedicine of Seville (IBiS, Spain). Raw data supporting the findings of this study are completely available from the corresponding author (MSS) on request.

3. Results

3.1. TAMs are a major source of Gal-3 in the TME of BCBM and GB

Firstly, we investigated whether the TME of BCBM and GB showed significant levels of Gal-3 and, was closely related to TAMs. The *in vivo* EO771 (Fig. 1A–C, BCBM) and GL261 (Fig. 1D–F, GB) models showed a strong presence of Gal-3 in the TME. Using Iba-1, a pan-marker of TAMs, we observed that most Gal-3 expression colocalised with these cells. Furthermore, to validate the Gal-3 KO (Fig. 1C and F) model, no presence of Gal-3 was found, except for a negligible amount likely derived from the tumour cells (Fig. 1A and D).

We also aimed at deciphering whether Gal-3 (LGALS3) expression had a prognostic value in breast cancer patients. Thus, after using The Cancer Genome Atlas (TCGA) database, we found that high expression of LGALS3 was significantly linked with poorer survival (Fig. 1G). Furthermore, human brain resections from patients with BCBM (Fig. 1H) had a strong expression of Gal-3 in the tumour foci, with clear increase in the core compared with the margins of the tumour. That Gal-3

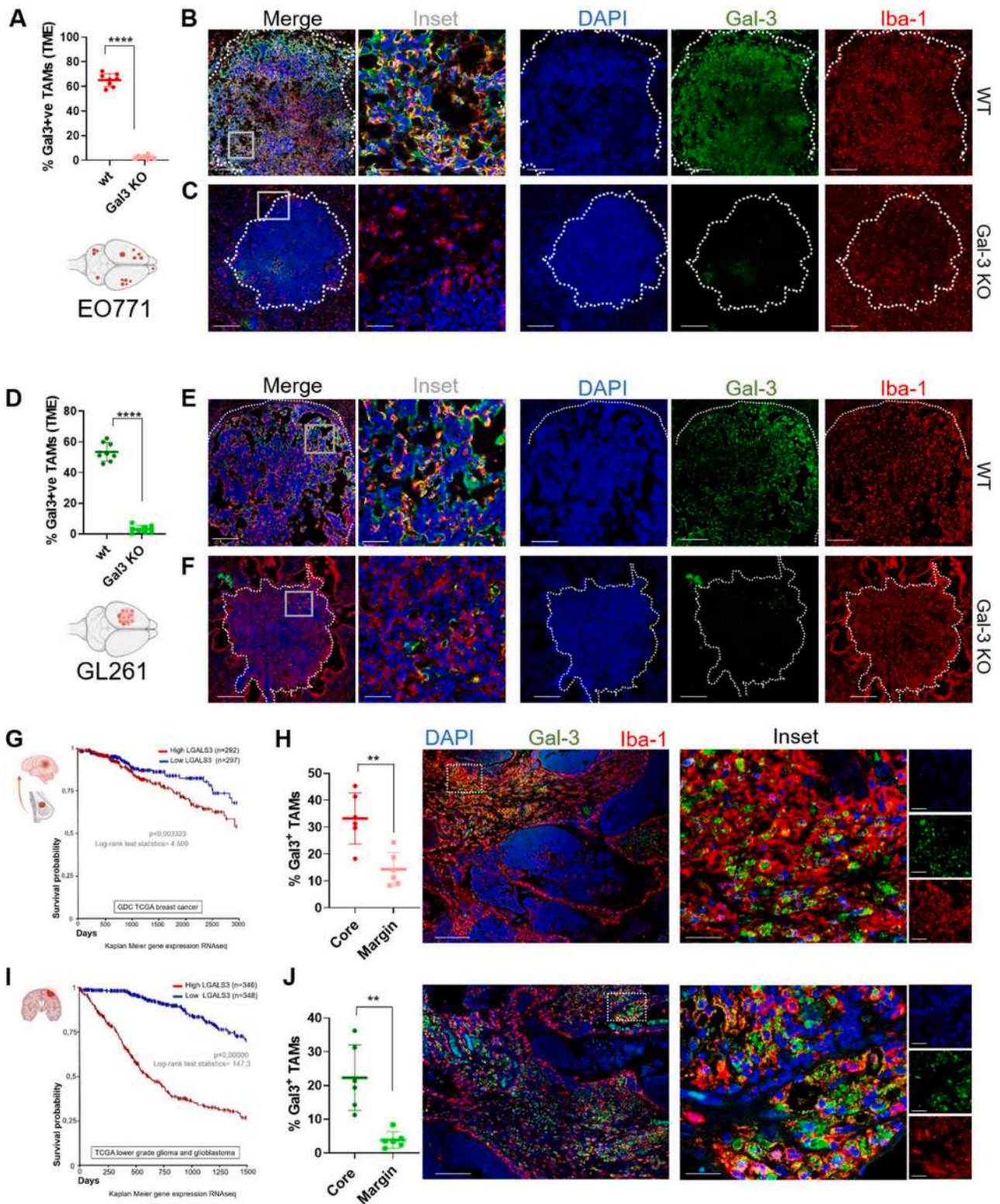


Fig. 1. A.-) Quantitation of TAMs expressing Gal-3 in the TME in wt and Gal-3 KO mice (n = 8) injected with EO771 cells. B.-) Immunofluorescence (IF) image (merge –left- inset –right-) showing the metastatic area in a wt mouse. (DAPI, blue), Gal-3 (Green) and TAMs (Iba1, red). C.-) Metastatic area of a Gal-3 KO mouse. D.-) Quantitation of TAMs expressing Gal-3 in the TME in wt and Gal-3 KO mice (n = 8) injected with GL261. E.-) IF image (merge –left- inset –right-) showing the GB area in a wt mouse. F.-) Metastatic area of a Gal-3 KO mouse. G.-) GDC TCGA breast cancer database analysing the deleterious impact of high levels of LGALS3. H.-) Quantitation (n = 6) of Gal-3-expressing TAMs. BCM resection from a 59-years-old woman, showing a strong presence of TAMs (Iba1, red) colocalising with Gal-3 (green). I.-) TCGA database showing the striking negative influence of high levels of LGALS3 in low and high-grade glioma patients. J.-) Quantitation (n = 6) of Gal-3-expressing TAMs. GB resection from a 67-years-old man, highlighting (inset) the area with strong presence of TAMs (Iba1, red) and Gal-3 (green). Scale bars: 100µm. *, p < 0,0001 (Welch’s t-test).

expression was closely related with TAMs and tumour cells, amongst other cell types. Regarding TCGA studies in GB samples, high LGALS3 was also closely linked with meagre survival in low grade and glioblastoma patients, suggesting the deleterious impact that this protein in those patients (Fig. 1D). Again, Gal-3 presence was found in GB resections, with the same pattern found in BCMB samples (Fig. 1J). In general, the heterogeneous presence of Gal-3 in the TME, likely forming areas of pseudopalisades, underscores its involvement in the adaptive strategies of tumour cells and TAMs within the stressed TME (e.g. nutrient deprivation or hypoxia). Altogether, with these results, we demonstrated a strong presence of Gal-3-expressing TAMs in two different forms of brain tumours.

3.2. Gal-3 triggers an anti-inflammatory phenotype in microglia cell line

Previous works from our research team described the key role of Gal-

3 in TREM2-associated microglial activation [7,11,19]. That phenotypic change in microglial response upon Gal-3 presence strongly suggested the need to elucidate the potential role of this lectin in the activating phenotype in TAMs.

3.2.1. EO771-conditioned medium

We aimed at deciphering how breast cancer-derived Gal-3 may regulate microglia polarisation. To this end, we monitored iNOS, Arg1, CD206 and PD-L1.

iNOS, the prototypic marker of pro-inflammatory microglia [20] (Fig. 2A), suffered a significant drop when soluble Gal-3 was added to the medium. Inversely, Arg1, CD206 and PD-L1 (Fig. 2B–D), prototypic markers of anti-inflammatory (pro-tumoural) microglia [6,21,22], once TCM was added, their expression was significantly increased. However, when Gal-3 was silenced in tumour cells and their conditioned medium was then added (Gal3KD), levels of every marker significantly dropped.

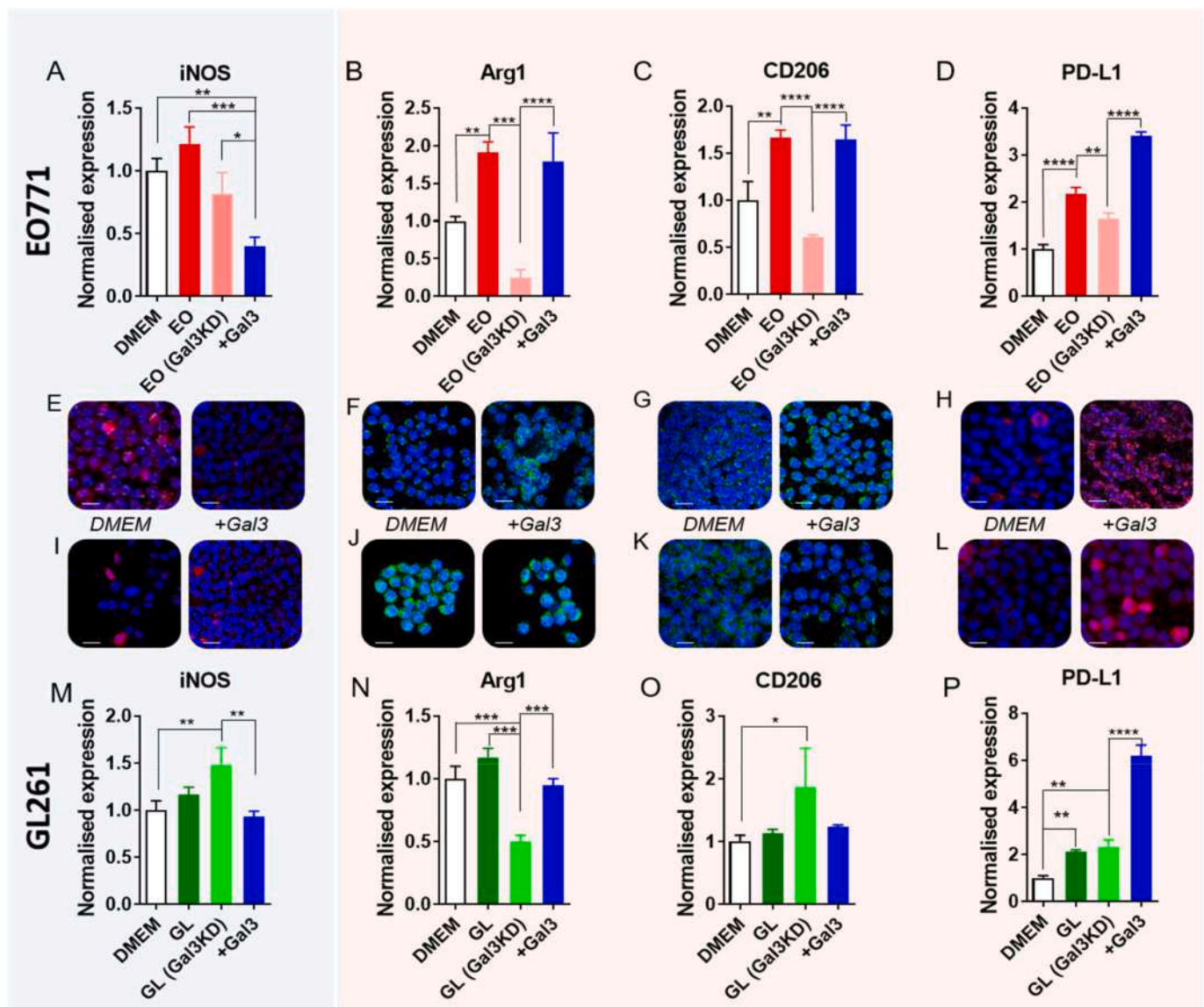


Fig. 2. Top row is devoted to BV-2 cells treated with EO771-conditioned medium. Bar graphs show the quantitative analysis of pro-inflammatory marker iNOS (A), and immunosuppressive Arg1 (B), CD206 (C) and PD-L1 (D). Cells were treated (24h) with tumour-conditioned medium from parental EO771 (EO) or EO771 cells with Gal-3 knockdown (siRNA, Gal3KD). Last experimental condition was used to study the effect of the exogenous addition of Gal-3 to EO-Gal3KD medium. Confocal Images showing the expression of iNOS (E, red), Arg1 (Green, F), CD206 (Green, G) and PD-L1 (H, red). Left images refer to control (DMEM) and right images to cells treated with exogenous Gal-3. As per EO771 group, Bottom row is devoted to BV-2 cells treated with GL261-conditioned-medium. Confocal images show expression of iNOS (red, I), Arg1 (Green, J), CD206 (K, Green) and PD-L1 (L, red). Bar graphs show quantitative analysis of the expression of iNOS (M), Arg1 (N), CD206 (O) and PD-L1 (P). Scale bars: 50 μm.

In contrast to iNOS, when exogenous Gal-3 was added back to the medium, the levels of every immunosuppressive marker were upregulated. Representative images of the above-mentioned markers are depicted in Fig. 2E–H.

At a gene level, iNOS showed a similar behaviour as previously described, since its gene expression was significantly upregulated (>3-fold) after EO-TCM and Gal-3 exposition. Similarly, Arg-1, CD206 and PD-L1 were upregulated once BV2 were exposed to TCM and Gal-3 (Supp. Figure 3).

Regarding the study using BV-2 Gal-3 KO cells, these cells were less responsive to all experimental challenges (Supp. Figure 4). However,

iNOS was downregulated when exogenous Gal-3 was added. Inversely, Arg1, CD206 and PD-L1 were upregulated upon Gal-3 addition, mimicking the previous results found with parental BV-2 cells.

All these data support the role of Gal-3 as a critical promoter of an anti-inflammatory phenotype in microglia, by upregulating classical immune-suppressive markers.

3.2.2. GL261-conditioned medium

When TCM from Gal-3 KD tumour cells was added (Gal3KD), iNOS was significantly increased in BV-2 (Fig. 2M). Interestingly, when Gal-3 was added, iNOS dropped to control levels. Conversely, the absence of

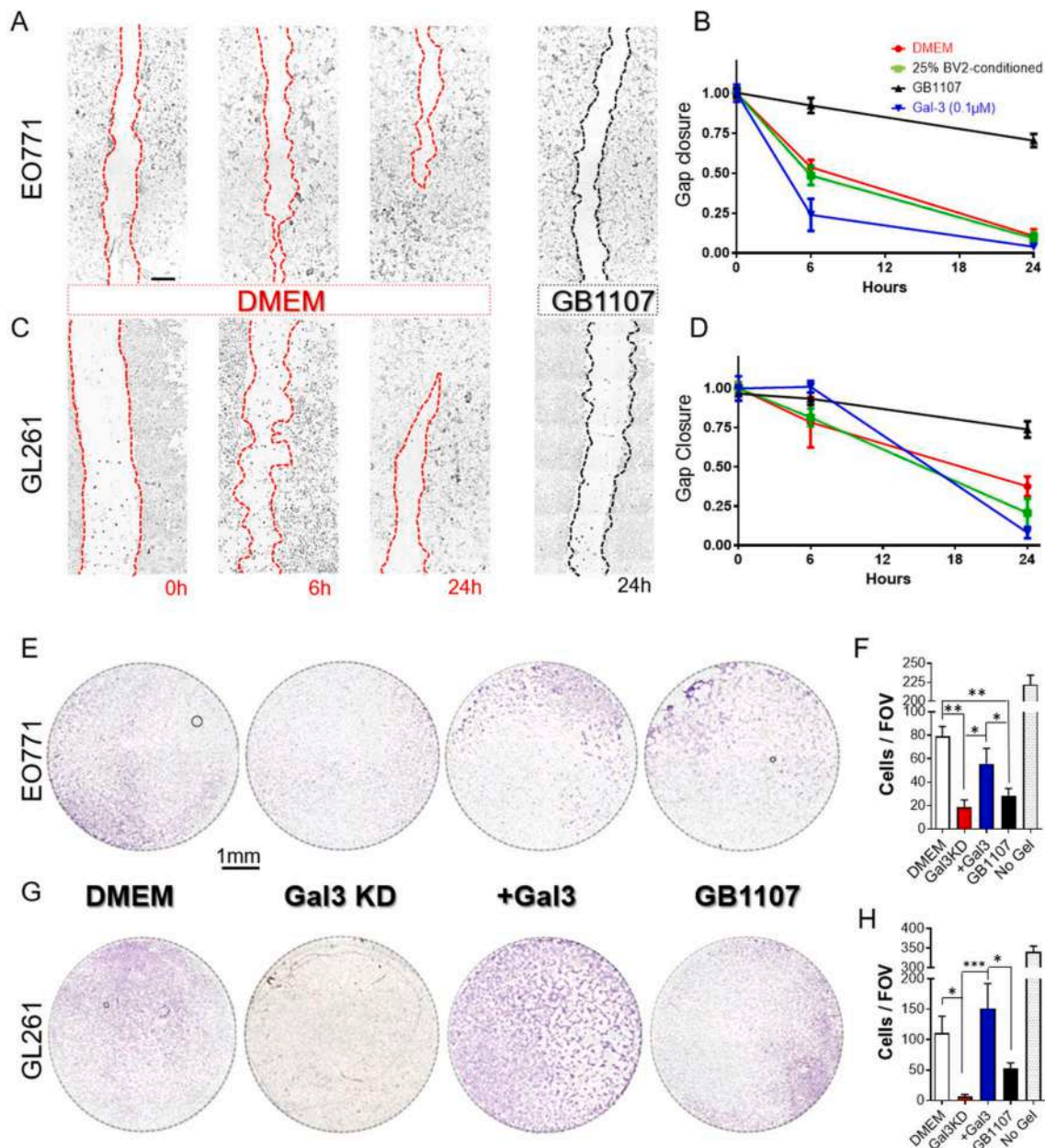


Fig. 3. A.-) Scratch-wound assay performed with EO771 cells in DMEM (red dotted lines) conditions. Time-course study with pictures at 0, 6 and 24h. EO771 cells after 24h exposition to GB1107 (black dotted line). B.-) Graph measuring gap closure ability of cells at different experimental conditions: control (DMEM, red line), microglia-conditioned medium (BCV2, Green line), selective Gal-3 inhibitor (GB1107, black line) and after addition of exogenous Gal-3 (blue line). C.-) Pictures of the scratch assay performed to GL261 cells in control (DMEM, red dotted line) and GB1107 condition (black dotted line). D.-) Quantitation of gap closure under the same conditions as described in B. E.-) Invasion assay performed in Boyden Chambers to EO771 cells, stained with crystal violet. Cells were coated with parental BV2 cells (DMEM) at the bottom well, or BV2 with Gal3 knockdown (siRNA -Gal3KD-). Next experimental condition was performed using exogenous Gal-3 with Gal3KD BV2 cells seeded at the bottom (+Gal3), and finally, using parental BV2 cells with GB1107 (0,1 μM). F.-) Quantitation of number of invading cells. G.-) Same as E, with GL261 cells. H.-) Same as F with GL261 cells. *p < 0,05; **p < 0,01; ***p < 0,005. One-way anova with Tukey post hoc analysis. Scale bar 750 μm.

Gal-3 in the TCM (Gal3KD) dropped Arg1 levels (Fig. 2N), whilst Gal-3 addition had an opposing effect, increasing Arg1 expression. Regarding CD206, its levels remained unaltered except when cells were coated with Gal3KD-medium (ca. 2-fold increase, Fig. 2O). This results may be expected since previous works showed that glioma cells upregulate CD206. Finally, PD-L1 was upregulated at all experimental condition (Fig. 2P), with special relevance upon Gal-3 addition (ca. 6-fold increase).

qPCR analysis showed how Gal-3 increased the gene expression of PD-L1 and Arg-1 even higher than the presence of TCM. In the case of iNOS, both Gal-3 and TCM inhibited its expression, thus, suggesting an anti-inflammatory behaviour in BV-2 cells upon Gal-3 presence (Supp. Figure 3)

BV-2 Gal-3 KO cells showed almost no phenotypic changes after the experimental challenges, suggesting the important role of endogenous Gal-3 to trigger the microglial response in the presence of TCM. Interestingly, PD-L1 was significantly upregulated when Gal-3 was added (Supp. Figure 4).

These data are in agreement with our previous findings (EO771-conditioned medium), highlighting the key role of Gal-3 upregulating classical immunosuppressive markers (e.g. Arg1 and PD-L1) in BV-2 [23].

Interestingly, we repeated the same experimental challenges in primary microglia cells, and the same trend toward the activation pro-inflammatory and the suppression of immunosuppressive markers was found [24,25] in cells from Gal-3 KO pups (Supp. Fig. 5 and Supp. Data).

3.3. Pharmacological blocking of Galectin-3 hinders tumour cells migration

We postulated that pharmacological blocking of Gal-3 (GB1107) [26], could restrain tumour cells migration. We used EO771 and GL261 to study their migrating properties in response to the cell permeable GB1107.

Interestingly, both cell lines (Fig. 3A–D) showed similar sensitivity to Gal-3 modulation. Both tumour cells exposed to microglial-conditioned medium (BV-2, Fig. 3B and D, green line) had similar gap closure speed compared with control (DMEM) conditions (Fig. 3A–D, red line). A slight increase in the migration pattern was observed when 0.1 μ M soluble Gal-3 (Gal-3, Fig. 3B–D, blue line) was added. Importantly, when both tumour cell lines were exposed to GB1107, their migration was greatly reduced (Fig. 3A–D, black line). Overall, our results sustain the relevant role of Gal-3 in tumour cell migration.

3.4. Microglial Galectin-3 supports tumour cell invasion

We aimed at studying the role of microglial Gal-3 in the invading properties of EO771 and GL261 cells. Performing an invasion assay with an artificial basement membrane [27], tumour cells were co-cultured with parental BV2 cells, and BV2 cells with Gal-3 expression knocked-down (Gal3 KD) (Supp. Figure 1B). EO771 cells that reached the luminal side of the membrane was significantly lower with BV2 Gal-3 KD cells (Fig. 3E–F and Supp. Figure 1C, Gal3KD). Adding back exogenous Gal-3 had a significant increase in the number of invading cells (+Gal3). The use of GB1107 (0.1 μ M), mimicked the results from Gal-3 KD condition, and successfully reduced the number of invaded tumour cells (Fig. 3E–F, GB1107).

Similarly, GL261 cells mirrored results found in EO771 cells (Fig. 3G). When coated with Gal-3KD BV-2 cells (Gal3KD), number of invading cells dropped ((Fig. 3G–H). Again, when Gal-3 was added back to the medium (+Gal3), the number of cells reached control values (DMEM). Importantly, when GB1107 was added, the number of invasive cells significantly dropped, thus, confirming the important effect of Gal-3 regarding invading capacity of both tumour cell lines (Fig. 3H, GB1107). These results underscore potential role of Gal-3 regulating the TME, exerting a robust influence on tumour cells migration and

invasion.

3.5. Glioblastoma and breast cancer brain metastasis burden is reduced in Galectin-3 knockout mice

EO771 and GL261 cells were implanted in wild-type C57Bl/6 and Gal-3 KO mice (n = 8, per experimental condition), and the tumour burden was quantified at different time points (Fig. 4, 10 and 21 days).

3.5.1. Breast cancer brain metastasis

Since 99 % of breast cancer cases account for women [28], we focused our BCBM *in vivo* study in female mice. Evaluation of the metastatic burden within the brain of wt and Gal-3 KO mice remained similar at day 10 (Fig. 4A–B). However, at day 21, Gal-3 KO mice showed a significant drop of the metastatic burden (Fig. 4C–E, ca. 8-fold).

3.5.2. Glioblastoma

Similar to the BCBM model, GB size showed no difference at day 10 (Fig. 4F–G). Again, at day 21, the tumour size was significantly reduced in Gal-3 KO mice (Fig. 4H–J, ca. 5-fold).

3.6. Galectin-3 affects vascular density and angiogenesis in the TME

The role of Gal-3 in promoting angiogenesis in cancers outside the brain has been extensively studied [24]. Our findings show that Gal-3 KO mice exhibited reduced angiogenic vessels compared to wt (Supp. Figure 6). Furthermore, vascular density was significantly decreased in the BCBM model, while the number of blood vessels remained similar in the GB model. These findings highlight the potential impact of Gal-3 on angiogenesis and vascularisation in different brain cancer models.

3.7. Galectin-3 depletion shifted TAMs activation towards a pro-inflammatory phenotype

We quantified immunofluorescent colocalisation of key pro- and anti-inflammatory markers in TAMs. In both models, we clearly observed a phenotype shift towards a pro-inflammatory phenotype over immunosuppression (Fig. 5).

No differences were found at day 10 after tumour implantation in either model (data not shown). However, at day 21, in the BCBM model, the expression of iNOS within the TAMs population was increased from 15 % in wt animals, to 40 % in the Gal-3 KO model (Fig. 5A–C). Importantly, Arg-1-expressing TAMs were reduced 50 % in Gal-3 KO mice (Fig. 5D–F). Again, these findings suggest the important role of Gal-3 in driving the pro-tumoural TAMs phenotype.

With regard to the GB model, percentage of iNOS-expressing TAMs increased from 23 % (wt) up to 58 % (Gal-3 KO) (Fig. 5G–I). On the contrary, the strong immunosuppressive phenotype in wt mice (high expression of Arg-1), was significantly dropped in Gal-3 KO mice (4-fold decrease, Fig. 5J–L).

Understanding the complexity of characterising the TME, owing in great part to the broad number of potential pro-vs anti-inflammatory markers to be used, the iNOS/Arg-1 balance (Supp. Figure 7A) may suggest that after Gal-3 depletion, TAMs showed a shift towards an anti-tumoural activation by increasing pro-inflammatory iNOS and reducing the expression of immunosuppressive Arg-1 [15]. Interestingly, the iNOS/Arg-1 balance in total number of cells (beyond TAMs) was tilted towards the pro-inflammatory side (Supp. Fig. 7). Further supporting the anti-tumoural scenario generated by TAMs in Gal-3 KO mice, CD68-positive cells were increased in the BCBM Gal-3 KO model, suggesting that Gal-3 depletion might enhance phagocytic features in TAMs. (Supp Fig. 8).

Lastly, in alignment with our previous findings, we employed GB1107 (28), to investigate its potential impact on TAMs phenotype. Given that brain metastases grow in close proximity to cerebral blood

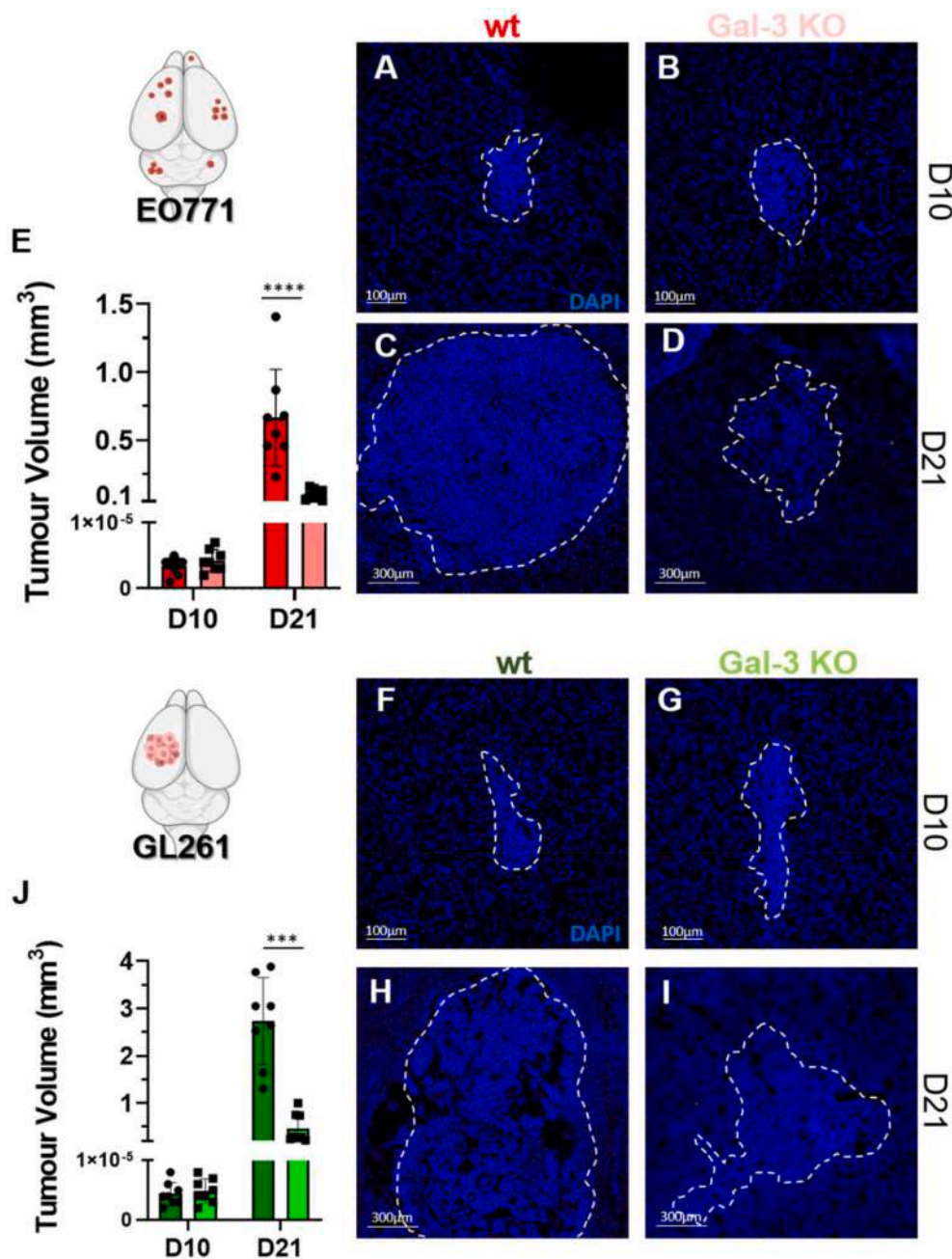


Fig. 4. Confocal images of the metastatic area of wt (n = 8) (A) and transgenic Gal3KO (n = 8) (B) female C57Bl/6 at day 10 and day 21 (C and D). E.-) Quantitation of the metastatic area in wt (red) and Gal-3 KO mice (light red bars). Confocal images of glioblastoma area in wt (F) and Gal3KO (G) mice injected with GL261 cells at day 10. H and I are images taken at day 21 post injection. J.-) quantitation of glioblastoma area comparing wt (dark Green) and Gal3KO mice (light Green) at 10 and 21 days. Statistical analysis of tumour volume progression was made independently across both times. One-way anova and Tukey post hoc tests was performed. ***, $p < 0,005$. ****, $p < 0,001$.

vessels, we aimed to determine whether GB1107 could shift TAMs behaviour towards a pro-inflammatory state. Remarkably, despite no significant reduction in brain metastasis size (data not shown), the TAMs phenotype in GB1107-injected mice mirrored our results from Gal-3 KO mice. We observed a significant increase in iNOS and a downregulation of Arg-1 across the TAMs population (Supp. Fig. 9). These findings suggest that GB1107 has the potential to modulate TAMs phenotype in the TME.

3.8. Gal-3 KO mice showed a robust anti-tumour T cell infiltration

Tumour-infiltrating lymphocytes (TILs) are also important

components of the TME. High level of CD4⁺ TILs combined with low CD8⁺ TILs is associated with poor prognosis in brain tumours [28]. Since Arg-1 expressing TAMs impair T cell responses inducing immunosuppression [15], we pursuit the potential effect of Gal-3-dependent TAMs polarisation in TILs population.

In BCBM, the intratumoural CD4⁺ T cells infiltration was significantly increased in wt mice (Fig. 6A–B). Importantly, CD8⁺ T cells infiltration had an opposing pattern and were more abundant in Gal3 KO mice (Fig. 6C–D). This CD8⁺/CD4⁺ T Cell ratio has long been described as a good prognosis marker in brain tumours [28].

In the GB model, TILs mirrored the results found in the BCBM mice. CD4⁺ T cells were less infiltrated in the Gal3 KO mice (Fig. 6F–G), whilst

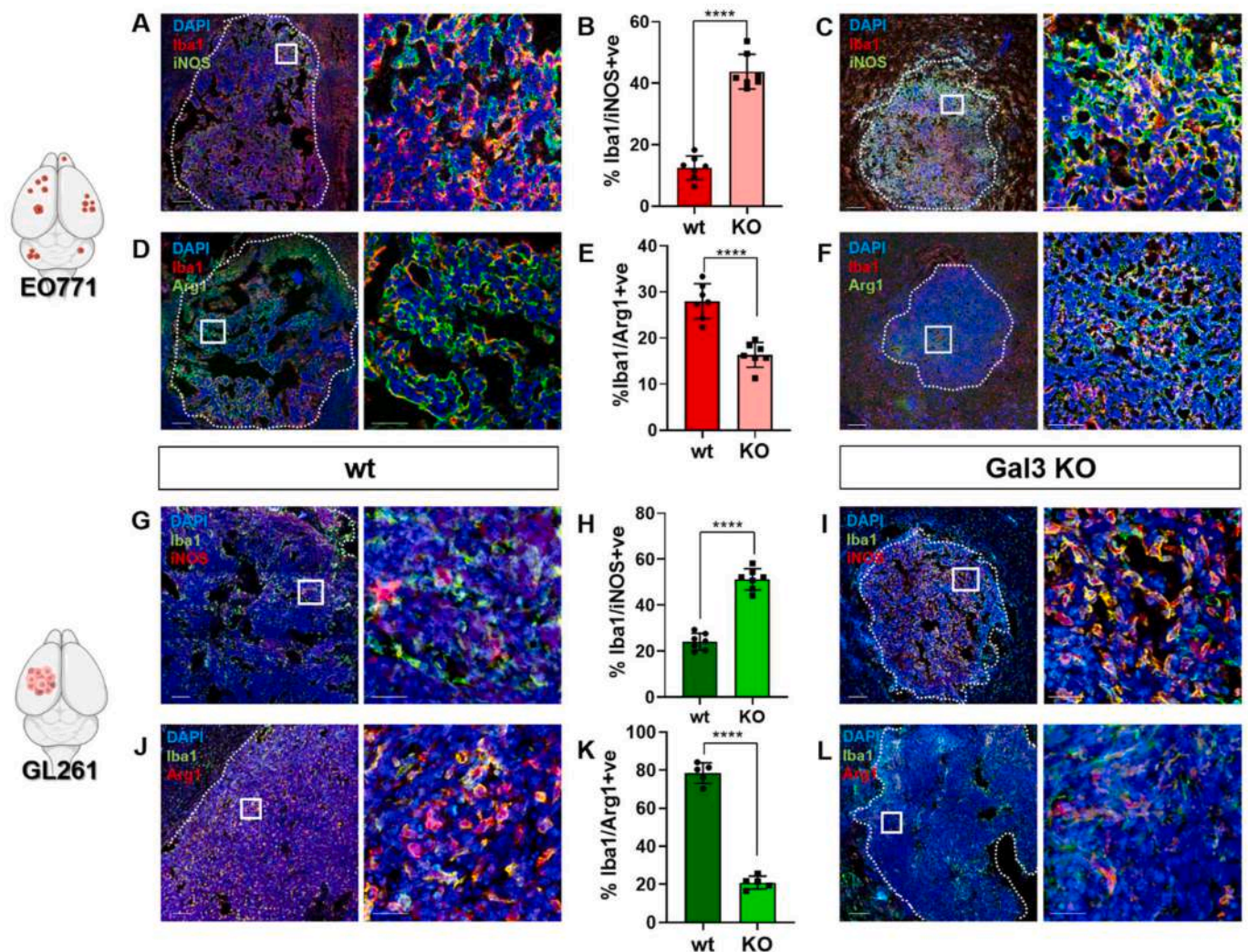


Fig. 5. A.-) Confocal images of the metastatic area of wt mouse at day 21 after being injected with EO771 cells. Pictures shows the TME (left image) and the inset (right) with nuclei stained in blue (DAPI), TAMs in red (Iba-1) and iNOS (green). B.-) Quantitation of TAMs expressing iNOS within the TME (n = 5). C.-) As per A, images showing the TME of Gal3KO mice. D.-) Same study as above-mentioned but analysing TAMs expressing Arg1 (E) in wt (D) and Gal3KO (F). G.-) Glioblastoma area of wt mouse injected with GL261 cells at day 21. Pictures shows the TME (left image) and the inset (right) with nuclei stained in blue (DAPI), microglia in green (Iba-1) and iNOS in red. H.-) Quantitation of TAMs expressing iNOS (n = 5). I.-) As per G, images showing the TME of Gal3KO mice. J.-) Same study as above-mentioned but analysing TAMs expressing Arg1 (K) in wt (J) and Gal3KO mice (L). Scale bars 250 μm (50 μm insets).

CD8⁺ T were significantly increased (Fig. 6H–I).

We observed a typical clustering pattern in TILs, presumably at sites of antigen presentation [29]. TAMs, as antigen-presenting cells, had a deep impact in TILs, since the pro-inflammatory phenotype found in Gal-3 KO mice, supported a tumouricidal CD8⁺/CD4⁺ TILs ratio (Fig. 6E and J).

3.9. Tumour size was further decreased after temozolomide treatment

Following the significant reduction in tumour size observed in Gal-3 KO mice, we sought to determine whether TMZ administration could potentially enhance the already described anti-tumoural microenvironment. Our findings revealed that animals treated with two rounds of TMZ exhibited a significant reduction in GB compared to those treated with TMZ alone (Supp. Figure 10). Moreover, our data indicate that TMZ shifted TAMs towards a pro-inflammatory state. Specifically, TMZ evoked an increase in iNOS expression in wt and Gal-3 KO mice, while Arg1 was significantly reduced in TMZ-treated Gal-3 KO mice (Supp. Figure 10). These findings suggest that Gal-3 deficiency may enhance the anti-tumoural effects of TMZ, which could have important

implications for GB treatment.

3.10. Gal-3 depletion enhanced a multifarious pro-inflammatory gene expression signature in the TME

In an initial effort to explore the pre-clinical relevance of our findings, we performed next generation transcriptome-wide gene-level expression profiling studies in the TME. Tumours from BCBM and GB animals were harvested at day 21 post-implantation, and then analysed.

3.10.1. Breast cancer brain metastasis

Comparing the TME of wt and Gal3 KO mice, we observed 180 dysregulated genes (100 genes upregulated and 80 genes down-regulated). The most of upregulated genes in the Gal-3 KO model were immune system-related, closely linked to pro-inflammatory markers (e.g. Tnfsf10, Tnfrsf1b), commonly expressed in microglia and macrophages (e.g. CD68, P2ry14), T cells (e.g. CD2, CD2, CD8b, Granzyme A-B) and Killer cells (Klrb1, KLR1a). On the contrary, wt animals showed pro-metastatic genes expression (e.g. Serpinb9f, Serpinb3b) [30] and arginase-1 [31] (Fig. 7A). Gene set enrichment analysis (GSEA)

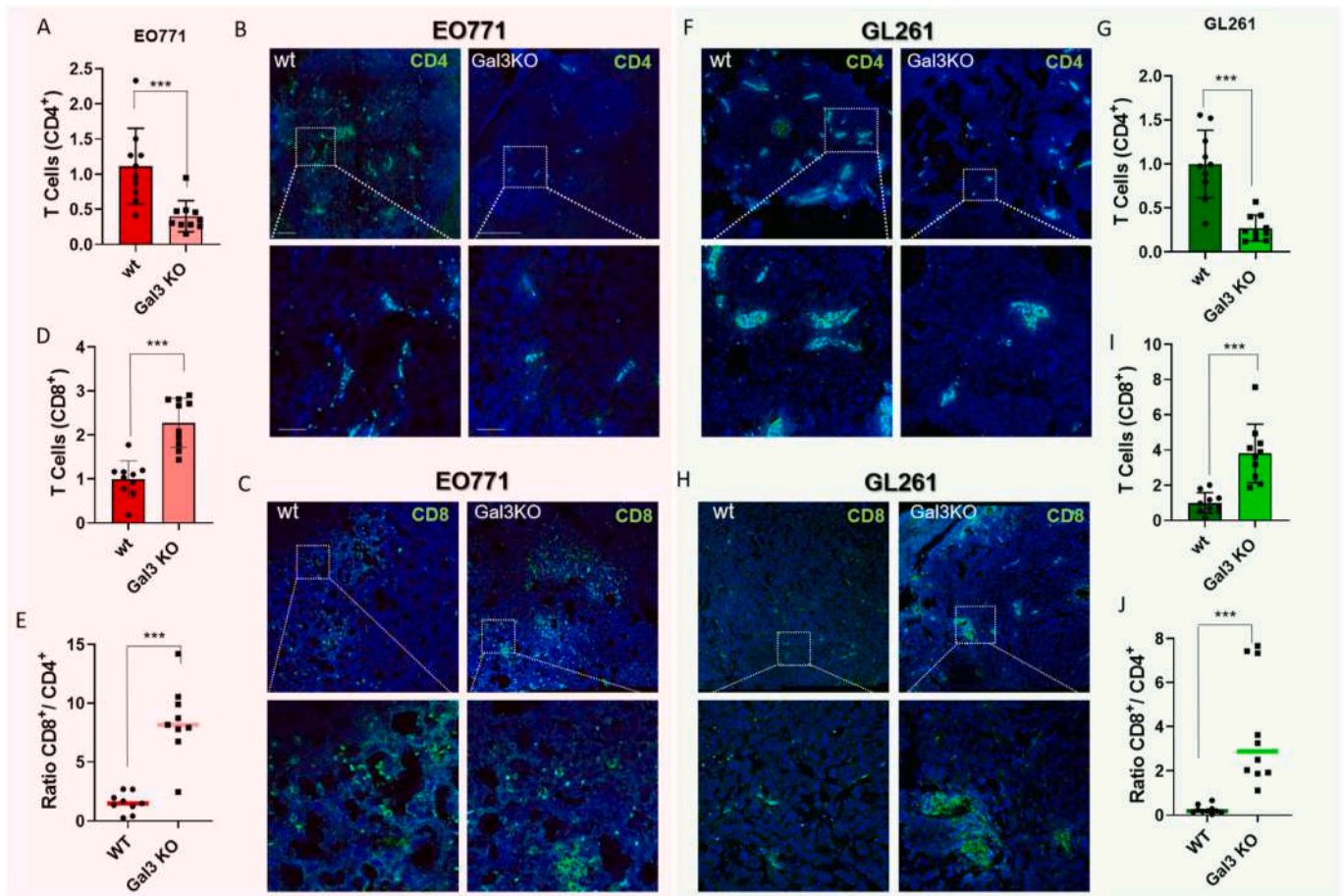


Fig. 6. A.-) Quantitation of CD4⁺ T cells within the TME (green) of BCBM. Nuclei in blue (DAPI) B.-) Confocal images of a BCBM showing CD4⁺ T cells (green) in the TME in wt and Gal3KO mice. C.-) Confocal images showing CD8⁺ T cells (green) in the TME of EO771 mice. D.-) Quantitation of CD8⁺ T cells within the TME of BCBM mice. E.-) Ratio of CD8 vs CD4 T cells within the TME of BCBM animals. F.-) Confocal images of CD4⁺ T cells in the TME of GL261-injected mice. G.-) Quantitation of CD4⁺ T cells (green) in the TME of GB model. H.-) confocal images of CD8⁺ T cells (green). I.-) Quantitation of intratumoural CD8⁺ T cells in our GB model J.-) CD8/CD4 T cells ratio within the TME of our GB model. Scale bar 250 μ m (100 μ m inset).

comparing over 22000 genes across both experimental models, showed several pro-inflammatory gene clusters in the Gal-3 KO model. Specially microglial response to interferon gamma was one of the most dysregulated clusters across groups (Fig. 7B–C), which is well-known to play a pivotal role in driving the pro-inflammatory phenotype in TAMs [32]. Moreover, using wikipathways analysis from TAC software, Gal-3 KO evoked an increase in different inflammatory gene signatures, with ADAR1 editing deficiency immune response and Type II interferon signalling as the most upregulated pathways (Fig. 7D). These pathways are mutually related since ADAR1 is a critical IFN-inducible form, displaying a pivotal role in driving pro-inflammatory responses of the immune system [33,34].

3.10.2. Glioblastoma

We observed 194 dysregulated genes, 111 were downregulated and 83 upregulated. Again, most dysregulated genes are typically expressed in activated immune system cells including microglia and macrophages (Clec7a, CD68, CD180) (Fig. 8A). GSEA showed the interferon gamma microglial pathway as one of the most upregulated clusters in the immunologic signature gene sets (Fig. 8B)

Using the Database for Annotation, Visualization and Integrated Discovery (DAVID) we observed that GO TERM biological processes showed chemokine-mediated pathways, together with interferon gamma and inflammatory responses (Fig. 8C). Finally, gene cluster analysis found upregulation in Viral protein interactions, which is closely related with ADAR-1 [34], and cellular response to interferon

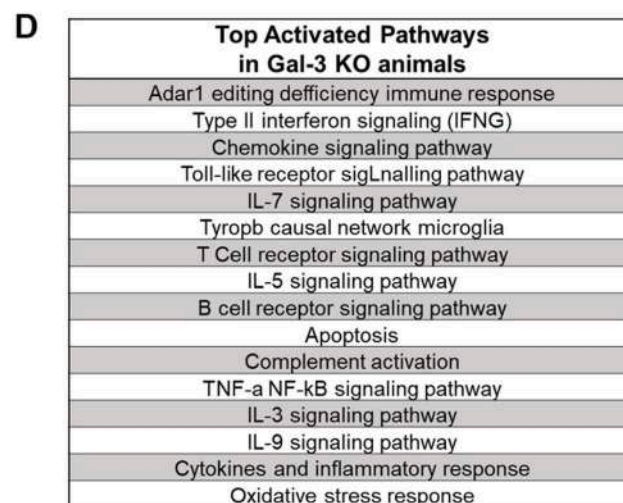
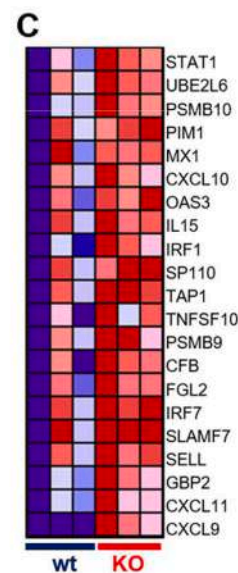
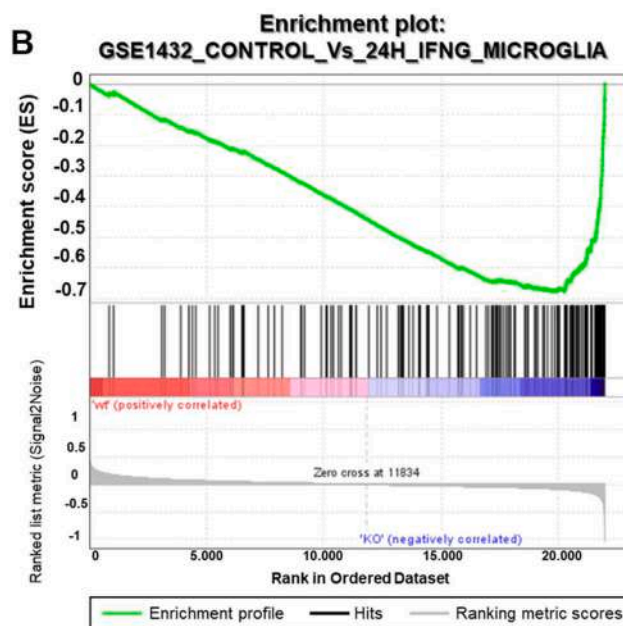
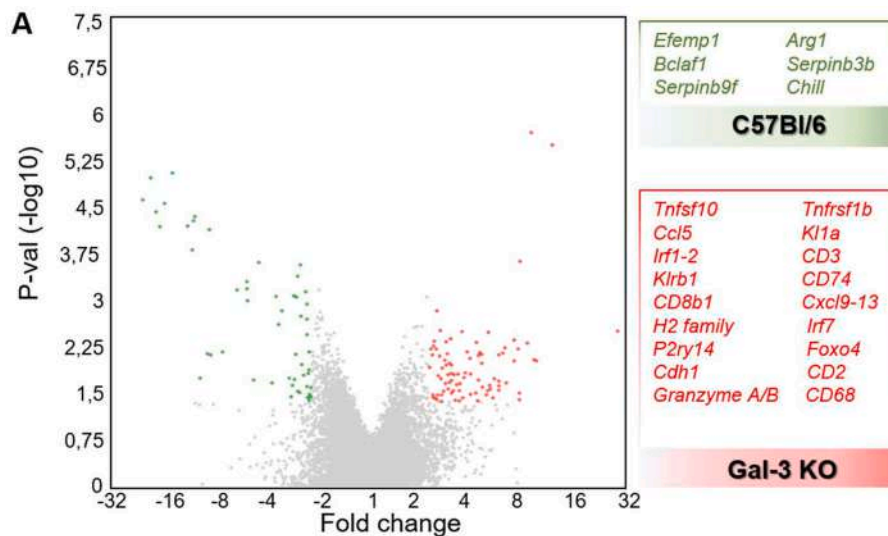
gamma (Fig. 8D).

Altogether, our transcriptomic analysis of the TME clearly show an immune-related pro-inflammatory microenvironment, which significantly affected BCBM and GB growth.

4. Discussion

Galectin-3 is a member of the evolutionary conserved animal lectins family. It is expressed in several cells types playing key roles in different biological processes such as apoptosis, autophagy, phagocytosis, inflammation, and cancer [8,35–38]. However, in the last years, our group has unmasked the pivotal role of Gal-3 in immune system cells activation, specifically microglia [7,19]. Thus, this work describes a novel role of this pleiotropic protein, activating an immunosuppressive phenotype in TAMs in a brain tumour context.

Gal-3 induces anti-inflammatory microglial activation in primary and immortalised microglia cell lines. We found a complex behaviour, underlining the activating effect that exogenous addition of Gal-3 exerted on classical immunosuppressive markers (e.g. Arg-1 and PD-L1), similar to the effects observed after the exposition to tumour-conditioned medium. Interestingly, such Gal-3-dependent anti-inflammatory response was abrogated once Gal-3 was inhibited. These results are in agreement with other works where blocking Gal-3 diminished levels of classical immunosuppressive markers [25,39]. However, our *in vitro* results also suggest that tumoural Gal-3 plays an important role driving such immunosuppressive microglial response. In particular, the



(caption on next page)

Fig. 7. A.-) Volcano plot showing genes with significant upregulation ($p < 0,05$ and <2 -fold change) in wt (green dots) and Gal3KO mice (red dots). Next to the plot there are two tables with previously reported pro-metastatic genes (green), which are upregulated in wt mice, and pro-inflammatory/immune system-related genes (red), upregulated in the Gal-3 KO mice. B.-) GSEA plot showing the upregulation of genes involved in the IFNG response in microglia, which was upregulated in Gal3KO mice. C.-) Heatmap showing the 20 top dysregulated genes in the microglial IFNG gene set. D.-) Most upregulated pathways in Gal3KO mice compared with wt.

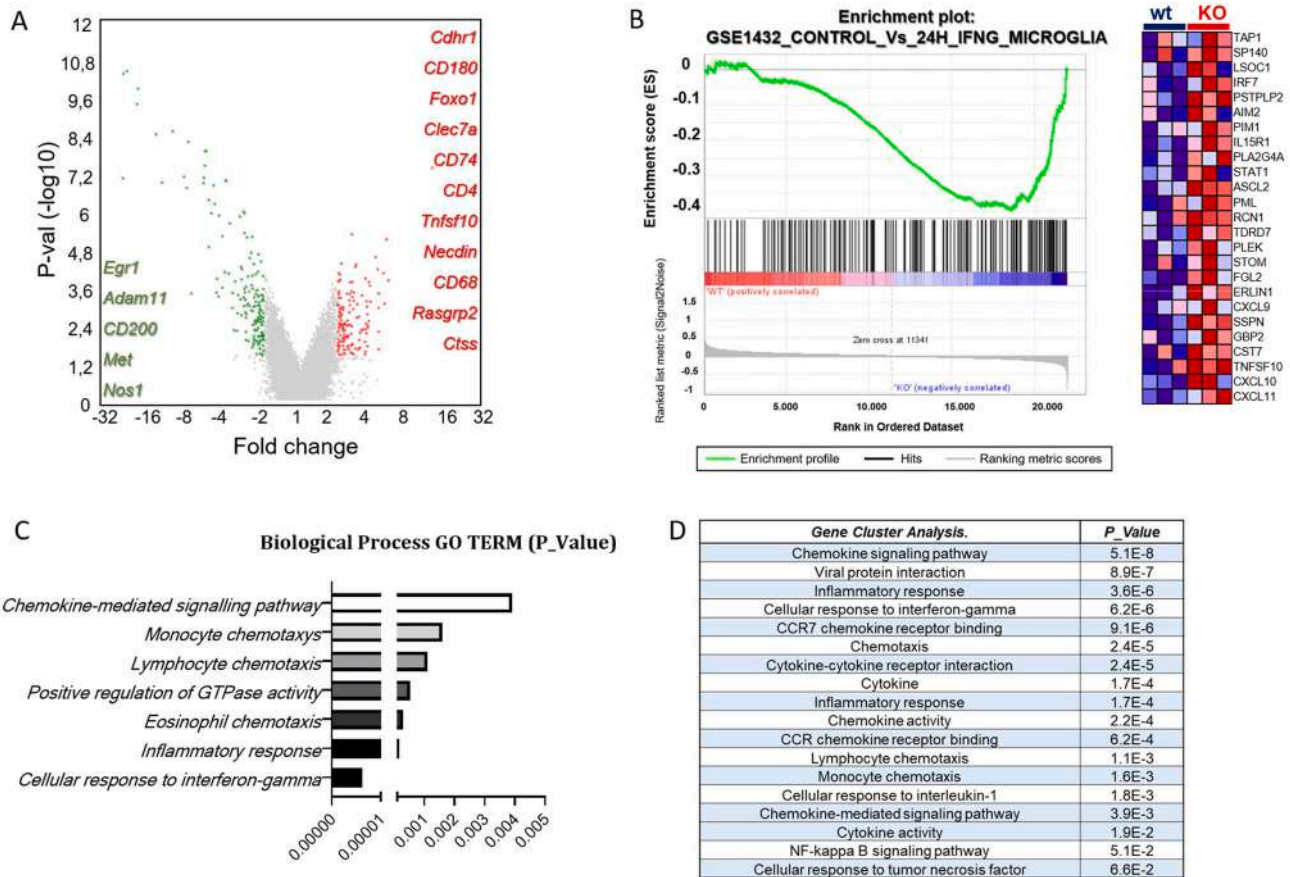


Fig. 8. A.-) Volcano plot showing genes with significant upregulation ($p < 0,05$ and <2 -fold change) in wt (green dots) and Gal3KO mice (red dots). There are two columns depicting genes with previously reported pro-tumoural features (green), which are upregulated in wt mice, and pro-inflammatory/immune system-related genes (red), upregulated in the Gal-3 KO mice. B.-) GSEA plot showing the upregulation of genes involved in the IFNG response in microglia, which was upregulated in Gal3KOs after using TAC software. Gene cluster analysis after using DAVID software, showing the most dysregulated pathways.

influence of Gal-3 in microglial PD-L1 expression is an appealing result since anti-PD-L1 therapies have experienced a great momentum in the clinic, with no great impact in patients' survival rates yet [26,40]. Concomitant target of Gal-3 together with anti-PD-L1 therapies, may offer an alternative option to overcome the pro-tumoural expression of this immune checkpoint inhibitor within the TME.

Furthermore, Gal-3 increases migration and invasion in different tumour types [9,24]. Interestingly, we have found how the use of GB1107 dropped migration speed in breast cancer and glioblastoma cells. These results are in accordance with previous works (in tumours outside the brain), where reducing the levels of Gal-3 showed a direct correlation with cancer cells motility and invasion reduction [41,42], although no specific dependence of microglial Gal-3 had ever been established. We have shown that microglial Gal-3 enhances the invasion properties of two different types of brain-invading cancer cells.

There are numerous pre-clinical works describing the role of Gal-3 promoting tumour growth and metastasis, predominantly in sites outside the central nervous system [9,41]. However, its role in brain tumours has been less studied. Using a full Gal-3 knockout transgenic mouse, we observed a significant drop of the tumour burden in BCBM and GB syngeneic models. Despite BCBM and GB are a heterogeneous

group of completely different diseases, we found a common pattern in both models: the TME in Gal-3 KO mice was shifted towards a pro-inflammatory state. TAMs population showed jointly a decrease in anti-inflammatory markers together with a significant upregulation of pro-inflammatory enzymes, hence, supporting their tumouricidal immune response. The activation of microglia is regarded as a double-edged sword, and the changes in either pro-inflammatory or anti-inflammatory effects mediated by Gal-3 depend on the disease types, stages and severity [43]. Thus, our study suggests that the immunosuppressive TME found in BCBM and GB could be partially reversed by increasing the population of iNOS-expressing TAMs and the suppression of the gold-standard immunosuppressive marker Arg1 [44] after Gal-3 inhibition. These findings place Gal-3 as a potential concomitant option to treat patients suffering from primary or secondary brain tumours. Actually, our results regarding the additive effect of tumour size reduction found in Gal-3 KO mice treated with TMZ further supports this hypothesis.

Our transcriptomic analysis of the metastatic microenvironment showed a clear upregulation of key important genes involved in pro-inflammatory (anti-tumoural) immune responses such as histocompatibility complex II, antigen presentation and, importantly, T cells. These

transcriptomic results were in agreement with our histological findings regarding the anti-tumoural CD8⁺/CD4⁺ TILs ratio. Furthermore, interferon and ADAR-1 pathways were also pathways clearly upregulated. Very recently, several studies have addressed the importance of ADAR-1 in promoting the immune response against tumours [34,45], highlighting the potential reach of our findings.

The transcriptomic analysis of the GB microenvironment also showed a clear upregulation of important inflammatory pathways in Gal-3 KO mice. Our research group previously established TREM2, primarily expressed in microglia, as a pivotal ligand of Gal-3 [19]. Recent studies have underscored the potential of TREM2 inhibition in enhancing anti-tumour activity within myeloid cells [32,46,47], thereby amplifying the efficacy of immunotherapies. In addition, the important role of RAGE as another ligand for Gal-3 has been recently described, thereby promoting glioma growth and invasion [48]. In contrast to the inhibition of TREM2 or RAGE, our findings reveal that exclusive Gal-3 inhibition yields similar effects in restraining glioma growth. This was achieved by promoting a pro-inflammatory response in TAMs and supporting anti-tumoural gene pathways (e.g. interferon-gamma).

In conclusion, this study furnishes compelling evidence highlighting the central role of Gal-3 in driving the polarisation of TAMs towards an immunosuppressive phenotype. This pro-tumoural state can be effectively reversed upon Gal-3 inhibition. Notably, for the first time, Gal-3 inhibitors are being explored for the treatment of central nervous system disorders such as Alzheimer's disease (NCT05959239). Therefore, Galectin inhibition has emerged as a promising avenue in cancer research [49], and our results could lay the foundation for novel pharmacological strategies aimed at enhancing the immune response in patients afflicted with primary or secondary brain tumours.

CRediT authorship contribution statement

Alberto Rivera-Ramos: Methodology, Investigation, Data curation. **Luis Cruz-Hernández:** Validation, Methodology, Investigation, Formal analysis. **Rocío Talaverón:** Writing – original draft, Supervision, Investigation, Data curation. **María Teresa Sánchez-Montero:** Methodology, Investigation, Data curation. **Juan García-Revilla:** Supervision, Methodology, Investigation, Data curation. **Marta Mulero-Acevedo:** Methodology, Investigation, Data curation. **Tomas Deierborg:** Writing – review & editing, Methodology. **José Luis Venero:** Writing – review & editing, Writing – original draft, Methodology. **Manuel Sarmiento Soto:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Manuel Sarmiento Soto reports financial support was provided by Ministry of Scientific Research and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2024.216879>.

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