

A topographic approach to the markers of macrophage/microglia and other cell types in high grade glioma

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ABSTRACT

In glioblastoma, glioma-associated microglia/macrophages (GAMs) represent the major population of tumor infiltrating cells, with up to one half of the cells of the tumor mass. Recent studies have shown that microglia are involved in the maintenance of immunological homeostasis and protection against autoimmunity. However, despite the growing body of evidence on the topic, many aspects are yet to be clarified. In our study, 3 different situations emerged concerning the markers of microglial/macrophage-related and other cell types in GBM patients: i) most of the markers (IBA1, TMEM119, CD206 and CD86) show an ascending gradient from the tumor center to the non-tumor/healthy area of the brain; ii) one marker (CD204) shows a descending gradient, going from the center of the tumor to the non-tumor/healthy brain area; iii) two markers (CD163 and P2RY12) show no gradient. These observations support the idea that the magnitude of the diverted inflammation is a 'extensive' rather than a 'local' phenomenon and that could possibly play a role in disease resistance and relapse.

Glioblastoma (GBM) represents one of the major challenges in modern oncology. Treatments have not significantly changed in the last 30 years: surgery remains the major therapeutic tool, while advancements in chemotherapy and immunotherapy have not met the objective of an improvement in survival (Bauman et al., 2022; Tan et al., 2020; Goodwin and Lattera, 2010; Minniti et al., 2023).

Evidence from the literature shows a crosstalk between GBM and the tumor microenvironment (TME), which has a dual role of active support of tumor progression and protection from immune system. TME includes a host of cell types, such as lymphocytes, resident microglia and recruited macrophages. In GBM, glioma-associated microglia/macrophages (GAMs) represent the major population, with up to one half of the cells of the tumor mass (Menna et al., 2022a; Manini et al., 2018). Bone-marrow-derived macrophages and monocytes account for 85% of total GAMs, while resident microglia accounts for the remaining 15% (Zhang et al., 2022). Recent studies have shown that microglia is involved in the maintenance of immunological homeostasis and protection against autoimmunity, in crosstalk with

dedifferentiated/stem-like glioblastoma cells, in migratory behavior with respect to infiltrative margins tumor cells and in activation patterns in the center of the tumor and the surrounding periphery (Zheng et al., 2022). In fact, a differential expression of GAMs was shown to significantly correlate with the biological behavior of tumors (Pearson et al., 2020). However, despite the growing body of evidence on the topic, many aspects are yet to be clarified. Questions are still open on GAMs distribution in the peritumoral area, on its spatial and molecular interaction with GBM infiltrative margins and with the stem cell compartment, and ultimately on the real extension of so-called TME with respect to tumor core.

GAMs can present with a wide range of phenotypes. It is generally agreed that the expression and release of proinflammatory cytokines and other mediators of inflammation, such as tumor necrosis factor (TNF), interleukin-1beta (IL-1β), and inducible nitric oxide synthase (iNOS) by activated macrophages and microglia is associated to an anticancer-targeted immune phenotype (M1 phenotype) (Zheng et al., 2022). Conversely, GAMs may play a role in the development of various

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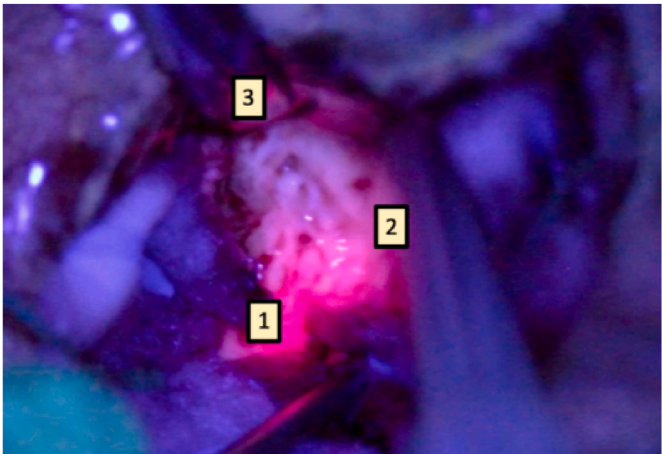


Fig. 1. Intraoperative sampling technique with 5-ALA under ultraviolet blue light (FL 400 filter during the resection of an High Grade Glioma. Dye accumulation is directly proportional to tumor cell concentration, it allows the distinction of three areas with different fluorescence levels, going from bright to pale to negative. Hence it is possible to identify (1) Tumor core with lava-like red fluorescence (ALA + area), corresponding to the enhancing area at MRI. (2) Tumor inner periphery with pale red fluorescence (ALA PALE±area), corresponding to the tumoral boundaries exceeding the MRI enhancing area. (3) Tumor outer periphery without fluorescence (ALA - area), corresponding to non-enhancing areas at a distance from the tumor.

characteristics of GBM, including expansion, invasion, proliferation, and immunosuppression. In particular, GAMs were shown to be able to switch to an M2 immunosuppressive phenotype, determining an anti-inflammatory environment that is associated to immune-escape and invasiveness, angiogenesis, and proliferation [(Zhang et al., 2022); (Pearson et al., 2020; Molina et al., 2019; Fan et al., 2021)]. However GAMs undergo a different pattern of activation depending on the stimulus; for example in a condition mimicking the late stage of pathology, microglia presents as a mixture of phenotypes (M1 and M2a/b), with up-regulation of iNOS (inducible nitric oxide synthase), ARG (arginase) and IL (interleukine)-10. At variance, in a condition resembling the early stage of pathology, GAMs shows a more specific pattern of activation, with increased M2b polarization status and up-regulation of IL-10 only (Lisi et al., 2014).

Currently, it is not clear if an activation occurs beyond the margins of tumor infiltration and, in the positive, to what extent; indeed, most observations are typically obtained from bulk-resected tissues, often non-specifically defined as tumor periphery or tumor core (Lisi et al.,

2017).

In the study presented here, we aim to perform a detailed analysis of specific markers within a topographic perspective, to understand if a ‘gradient’ of activation status exists with respect to tumoral core and beyond the radiologically evident tumor (Menna et al., 2022b; Della Pepa et al., 2020).

Therefore, a novel intraoperative sampling technique was introduced that is based on a GBM-specific fluorescent metabolic marker. The latter is tumor specific and directly proportional to tumor cell concentration, hence allowing a topographic distinction of different compartmental areas of tumor core, close and far periphery (Menna et al., 2022b; Della Pepa et al., 2020, 2023a, 2023b; La Rocca et al., 2020; La et al., 2019).

1. Materials and methods

1.1. Study design

Tumor tissue samples were collected from patients who underwent surgery for a histopathologically confirmed diagnosis of GBM at the Neurosurgical Department of Fondazione Policlinico Universitario Agostino Gemelli IRCCS (Rome, Italy). The ethic committees of the Fondazione Policlinico Universitario Agostino Gemelli (Prot. N. ID 2253) have approved the research. Written informed consents have been obtained from patients and all clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Only newly diagnosed GBMs were included. Exclusion criteria were as follows: needle biopsies, recurrent gliomas and second surgeries.

For each of the patients, the following parameters were extracted.

- Demographic: sex, age at diagnosis

Table 1
Indication of used antibodies.

Ab	HOST	Catalog	Company	Dilution
Iba1	goat	NB100-1028	Novus biologicals	1:250
CD204	mouse	14-9054-82	Invitrogen	1:1000
CD206	mouse	SC-376232	Santa Cruz	1:100
TMEM	rabbit	NBP2-30551	Novus biologicals	1:150
CD86	rabbit	91882	Cell Signaling	1:80
ARG1	rabbit	MBS179561	MyBioSource	1:100
CD163	mouse	AB156769	Abcam	1:500
P2RY12	rabbit	MBS244421	MyBioSource	1:150
iNOS	mouse	MAB9502	R&D Systems	1:200

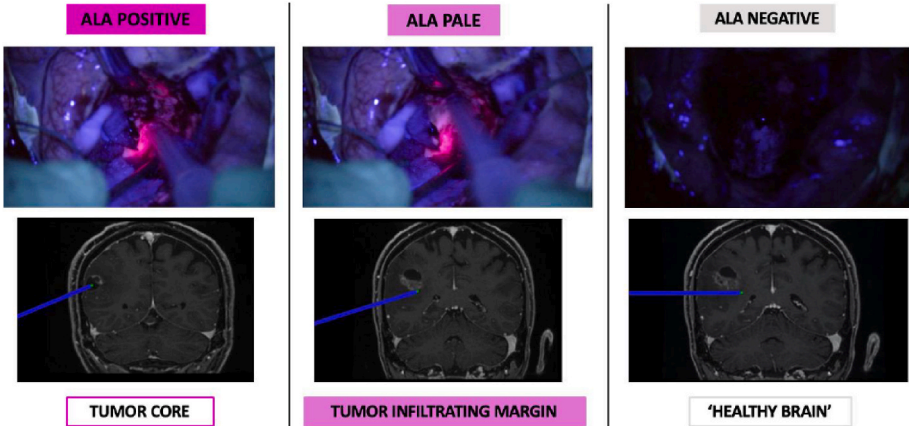


Fig. 2. representation of the sampling areas based on 5-ALA fluorescence compared to neuronavigation on T1w contrast-enhanced MRI. 5-ALA is a biological rather than a permeability marker - its fluorescence mark tumor tissue volume exceeding gadolinium enhancing volume at MRI. the ALA + area, grossly corresponds to the Gadolinium enhancing area the ALA±and ALA - areas extend beyond the limits of the MRI enhancing tumor.

Table 2
Sequences of used primers.

PRIMER	FORWARD	REVERSE	
GAPDH	5'-CCCTTCATTGACCTCAACTACA-3'	5'-TGGGATTTCATTGATGACAAGC-3'	
IBA1	5'-TCATGTCCTGAAACGAATG-3'	5'-CCAGCATCATCCTGAGAAAG-3'	136 bp
TMEM 119	5'-ACTTCTGTGGATGGGATAGTG-3'	5'-GGGAAGGACGATGGGTAATA-3'	155 bp

Table 3
Demografich characteristics of patients.

Codename	Age	Sex	Localization	Side	Ki67%	IDH1	MGMT met Status
1	21	F	Frontal	Left	30%	mutant	Methylated
2	84	M	Frontal	Left	20%	wt	Methylated
3	66	M	Parietal	Left	20%	wt	Methylated
4	56	M	Frontal	Left	2%	wt	Methylated
5	61	M	Frontal	Right	20%	wt	Methylated
6	56	F	Frontal	Right	10–15%	wt	Methylated
7	62	M	Occipital	Right	20%	wt	Methylated
8	74	M	Temporal	Left	15–20%	wt	Methylated
9	68	M	Frontal	Left	15–20%	wt	Methylated
10	71	M	Parietal	Left	20%	wt	Methylated
11	65	M	Temporal	Left	5%	wt	Unmethylated
12	77	M	Parietal	Left	30%	wt	Methylated
13	72	F	Temporal	Left	15–20%	wt	Methylated
14	70	M	Frontal	Right	25%	wt	Methylated
15	49	F	Frontal	Left	15%	mutant	Methylated
16	61	M	Temporal	Left	10–15%	wt	Methylated
17	70	F	Occipital	Left	20%	wt	Unmethylated

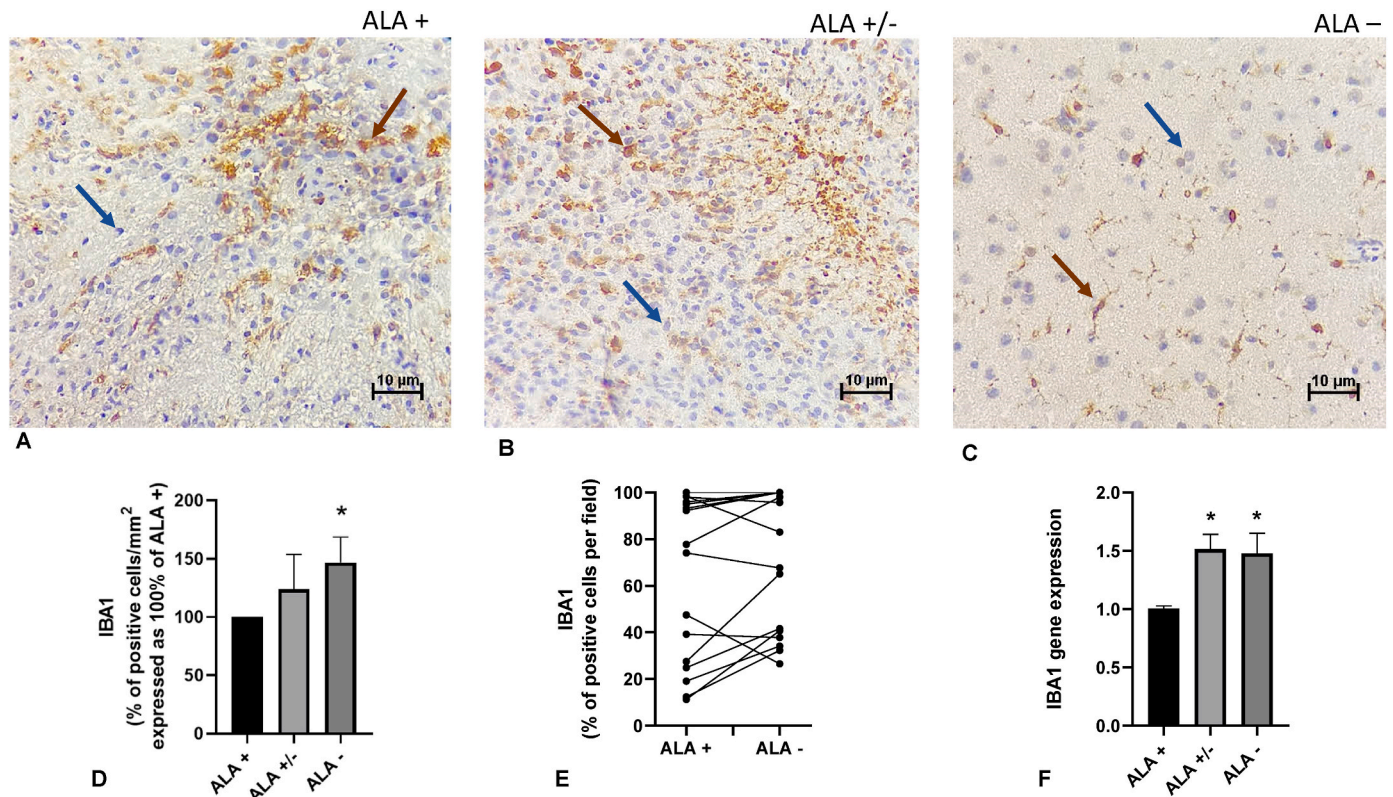


Fig. 3. IBA1 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for IBA1 (magnification: 10 ×). Brown arrows point to IBA1 stained cells, whereas blue arrows point to IBA1 negative cells, respectively. The histograms represent the cumulative (D), the individual (E) and gene expression (F) analysis. Data are expressed as mean ± SEM of samples collected from 17 patients. Tumor versus non-tumor/healthy area * $p < 0.05$.

- Radiological: tumor location, side, midline shift, ependymal and corpus callosum involvement, presence of necrosis/cystic area, tumor volume (measured on contrast enhanced T1), EOR
- Molecular: O-6-methylguanine-DNA methyltransferase (MGMT) methylation, isocitrate dehydrogenase 1 (IDH1) mutation, Ki67 proliferative index.

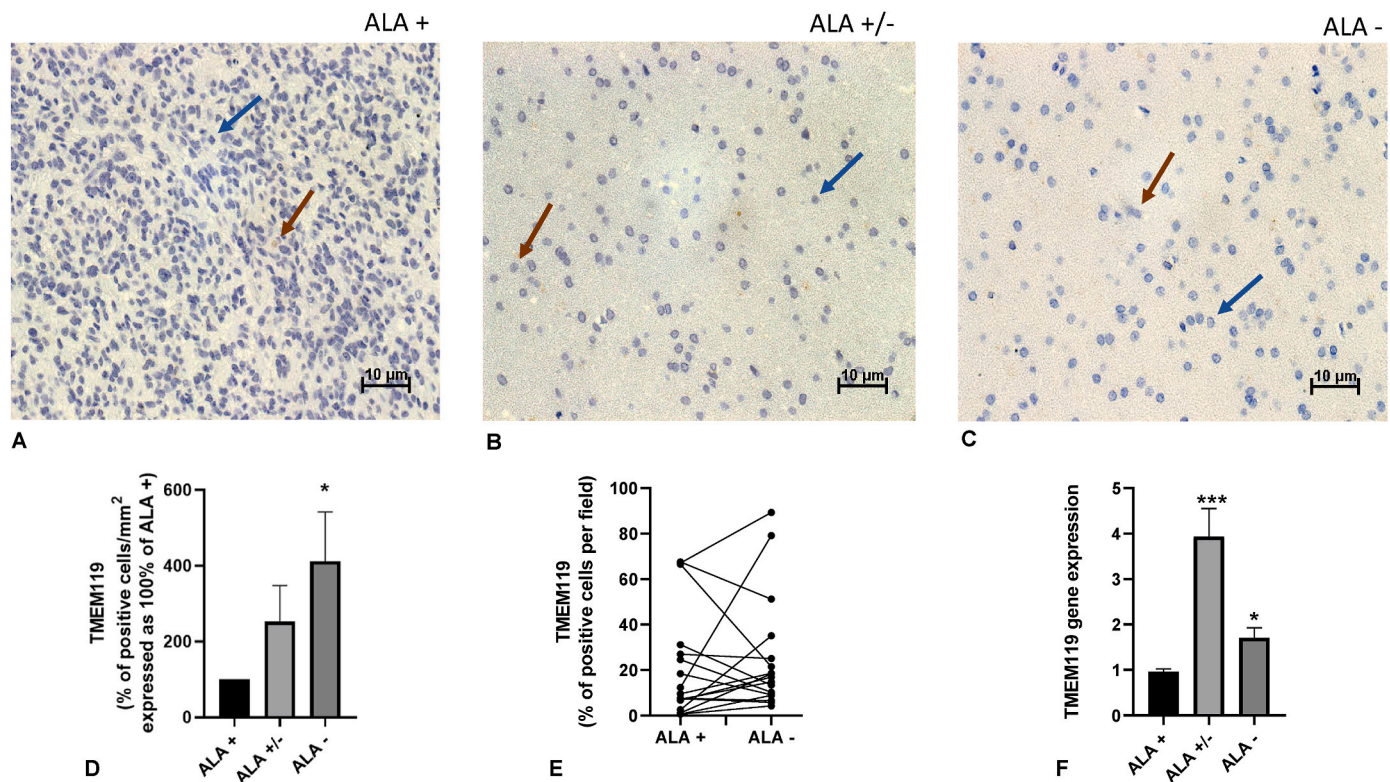


Fig. 4. TMEM119 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for TMEM119 (magnification: $10\times$). Brown arrows point to TMEM119 stained cells, whereas blue arrows point to TMEM119 negative cells, respectively. The histograms represent the cumulative (D), the individual (E) and gene expression (F) analysis. Data are expressed as mean $\pm$ SEM of samples collected from 17 patients. Tumor versus non-tumor/healthy area * $p < 0.05$.

The study is designed as follows: GBM samples were intraoperatively collected on the bases of the fluorescent metabolic marker that is tumor specific and directly proportional to tumor cell concentration. Per each case 3 samples were collected corresponding to different compartmental areas of tumor: core, close periphery (infiltrative margin) and far periphery (beyond tumor margin) as detailed in next paragraph.

1.2. Intraoperative fluorescence based sample collection

Samples were collected taking advantage of the fluorescence-guided surgical resection technique with 5-aminolevulinic acid (5-ALA). This fluorescent, tumor specific marker is widespread used in the neurosurgical setting of GBM surgery nowadays. 5-ALA (20 mg/kg body weight; Medac GmbH, Wedel, Germany) was administered orally to patients 3–5 h prior to neurosurgery. At the time of surgery, three different tumor macro-areas were visualized based on the presence of red-violet fluorescence, following excitation with ultraviolet blue light (FL 400 filter, Leica M720 OH5; Leica Microsystems, Wetzlar, Germany) (La Rocca et al., 2020; Della Pepa et al., 2023a, 2023b; La et al., 2019)

Sampling sites were categorized as follows (Figs. 1 and 2).

- (1) Tumor core with lava-like red fluorescence (ALA + area), corresponding to the enhancing area at navigation.
- (2) Tumor inner periphery with pale red fluorescence (ALA PALE $\pm$ area), corresponding to the tumoral boundaries directly surrounding the enhancing area.

- (3) Tumor outer periphery without fluorescence (ALA - area), corresponding to non-enhancing areas at navigation at a distance from the tumor.

Every sample site was co-recorded with intraoperative neuro-navigation (based on preoperative MRI on contrast enhanced T1w and FLAIR images).

A few mm of tissue were collected on solid non-necrotic areas and preserved in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 4 °C, before processing.

1.3. Immunohistochemistry on bulk tissue

The PT Link (Dako, Agilent Technologies, Santa Clara, CA, USA) was used to deparaffinize and rehydrate the sections and unmask antigen sites. Slides were immersed in 10 mM citrate buffer, pH 6.0, for 10 min at 97 °C and then cooled and washed in TBS (phosphate saline buffer). Endogenous peroxidase activity was inhibited by incubating the slides with Peroxide Block (ScyTek Laboratories, Utah, USA) for 7 min. After washing in distilled water and then in TBS, sections were treated with Avidin-Biotin Kit (Biocare-Medical Pacheco, CA, USA) to reduce background staining caused by endogenous biotin. After 3 washes in TBS, nonspecific binding was blocked by 10 min incubation with Background Punisher (Biocare-Medical). Sections were incubated overnight at 4 °C with primary antibodies, washed extensively in TBS and subsequently incubated with Ultratek HRP kit (ScyTek Laboratories) and the reaction was revealed by incubation with 3,3'-diaminobenzidine (Biocare-

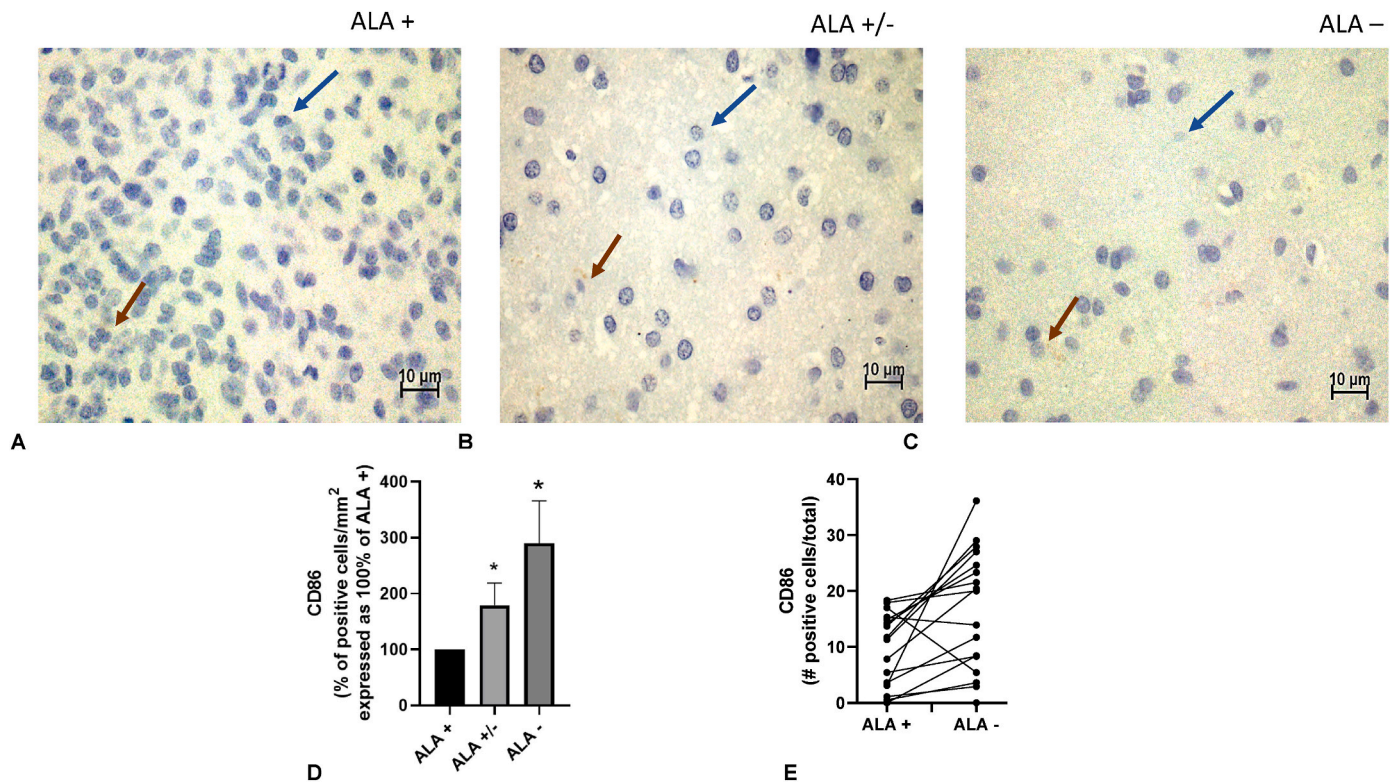


Fig. 5. CD86 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for CD86 (magnification: $10 \times$). Brown arrows point to CD86 stained cells, whereas blue arrows point to CD86 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean $\pm$ SEM of samples collected from 17 patients. Tumor versus non-tumor/healthy area * $p < 0.05$.

Medical). For quantitative analysis, two blinded examiners counted the number of positive cells per field. The data are expressed as the number of positive cells per square millimeter.

Table 1 reports the antibodies used.

1.4. Real-time quantitative RT-PCR (qRT-PCR)

Total RNA from cell was extracted using the Trizol reagent protocol following the manufacturer's instructions. RNA concentration was measured using the Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific). Aliquots (300 ng) of RNA were converted to cDNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by qRT-PCR using the following conditions: 35 cycles of denaturation at 95°C for 20 s, annealing and extension at 60°C for 20 s, and using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Stratagene, San Diego, CA, USA). PCR reactions were carried out in a 20-µL reaction volume in AriaMX real-time PCR machine (Agilent, Santa Clara, CA, USA). Gene expression was evaluated using the following primers (Table 2):

Relative mRNA concentrations were calculated from the take-off point of reactions (threshold cycle, Ct) using the comparative quantitation method provided by AriaMX software (Agilent Aria v1.5) and based upon the $-\Delta\Delta\text{Ct}$ method. Ct values for GAPDH expression served as a normalizing signal.

1.5. Statistical analysis

Data were described as median $\pm$ standard error of the mean (SEM). For multiple comparisons, one way ANOVA was used, followed by Dunnett's multiple comparisons post-test. Statistical significance was determined at the $\alpha = 0.05$ level. Differences were considered statistically significant when $p < 0.05$. Statistical analysis was performed with GraphPad Prism software version 7.04 (GraphPad Software, San Diego, CA).

2. Results

In Table 3 the demographic characteristics of patients are reported.

For each patient, three different areas were sampled (ALA+; ALA ± and ALA-) and several cell markers and/or activation factors were evaluated. For each marker two different analyses have been carried out: 1) cumulative analysis by placing the number of positive cells present in ALA + at 100% and reporting in % with respect to ALA + the number of positive cells in ALA ± and ALA -; 2) individual analysis for each patient showing the % of positive cells on the total number of cells counted in ALA+ and ALA -. The analysis considers the number of positive cells but not the intensity of staining.

Cumulative analysis for IBA1, a marker for microglia-macrophage cells, showed an increasing gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Fig. 3D): in the non-tumor/healthy area, the percentage of positive cells is significantly

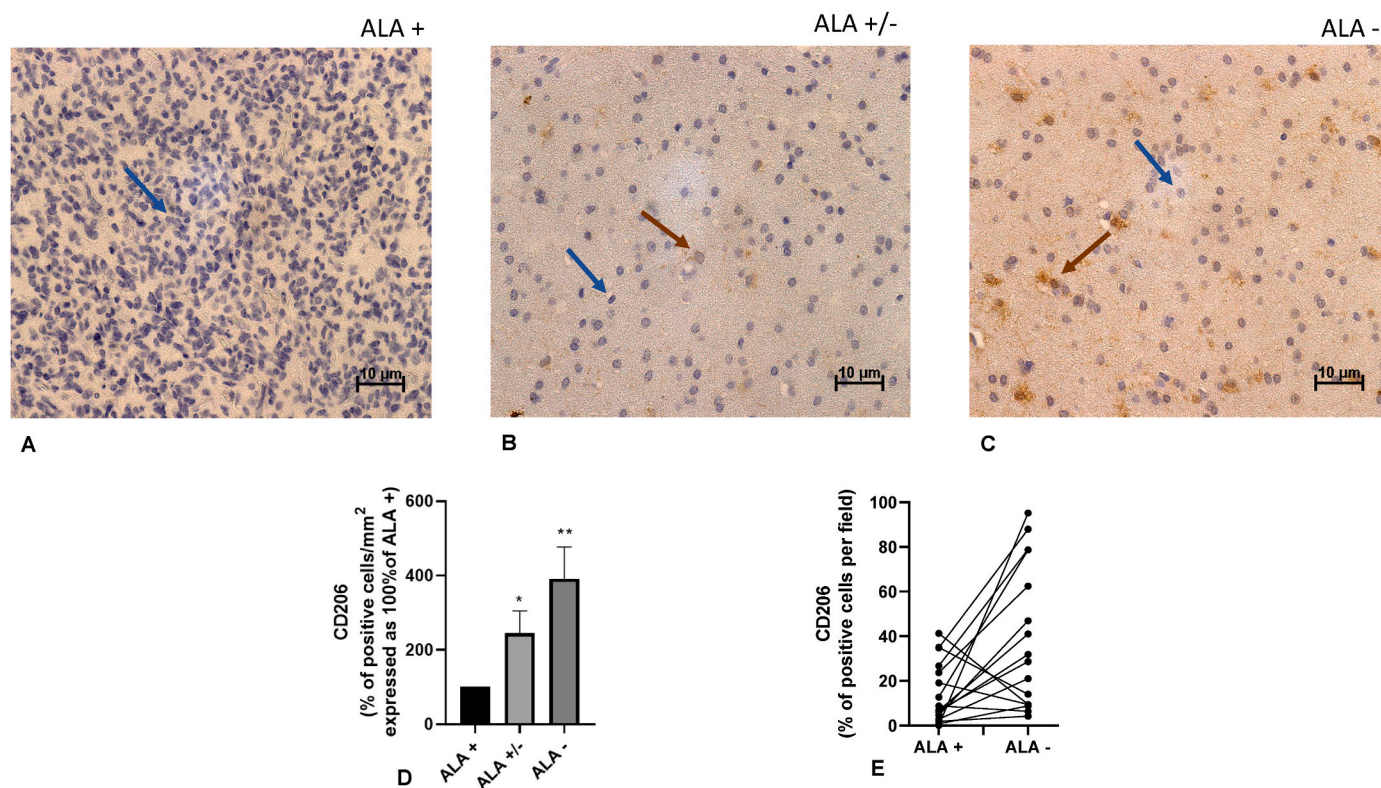


Fig. 6. CD206 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for CD206 (magnification: $10 \times$). Brown arrows point to CD206 stained cells, whereas blue arrows point to CD206 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean $\pm$ SEM of samples collected from 17 patients. Tumor versus non-tumor/healthy area * $p < 0.05$.

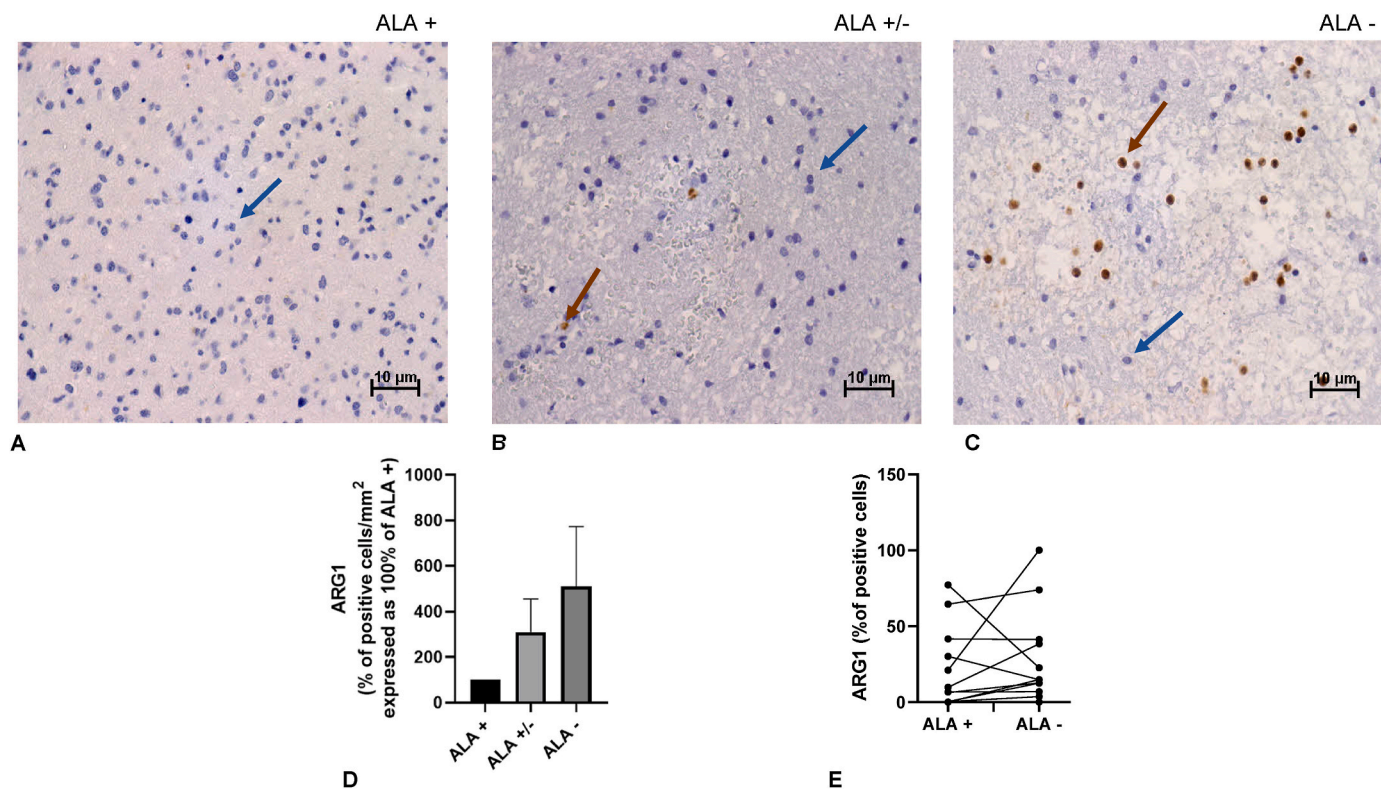


Fig. 7. ARG1 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for ARG1 (magnification: $10 \times$). Brown arrows point to ARG1 stained cells, whereas blue arrows point to ARG1 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean $\pm$ SEM of samples collected from 17 patients.

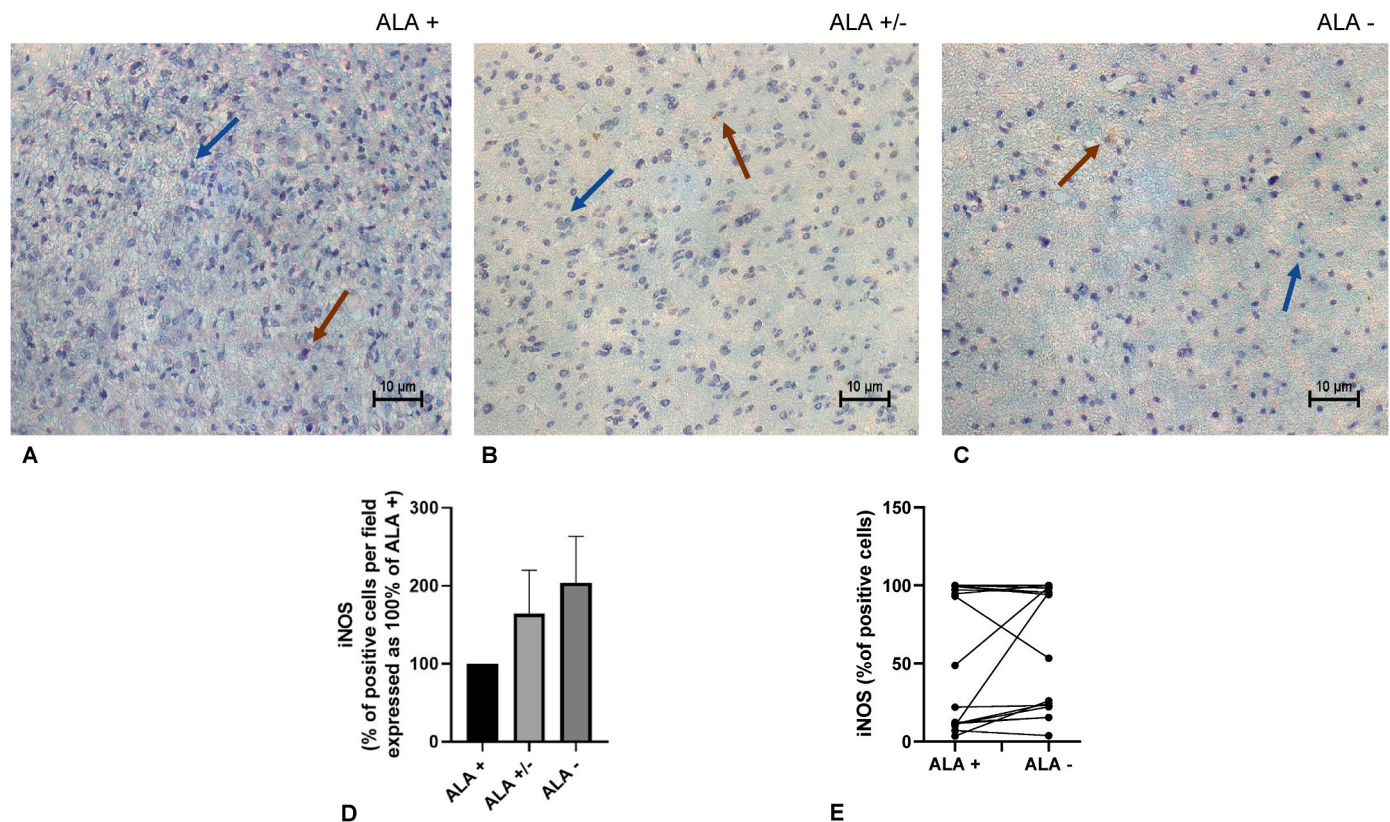


Fig. 8. iNOS staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for iNOS (magnification: $10 \times$). Brown arrows point to iNOS stained cells, whereas blue arrows point to iNOS negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean $\pm$ SEM of samples collected from 17 patients.

higher than the percentage of positive cells present in the tumor center. Consistent analysis of individual patients showed that 12 (70%) out of 17 patients had an increase in the number of IBA1-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 3E). As previously published (Lisi et al., 2017), the study of different GBM brain areas using IBA1 has highlighted 2 different cell morphologies: positive IBA1 cells with branched morphology prevalent in ALA - (Fig. 3A), and positive IBA1 cells with amoeboid morphology, prevalent in ALA + and ALA +/- (Fig. 3B and C). Accordingly, the evaluation of the RNA extracted from the tissue of 3 examined areas also showed a significant increase in IBA1 gene expression in the non-tumor area compared to tumor core (Fig. 3F).

Similarly to IBA1, cumulative analysis for TMEM119, another marker for microglia/macrophages but also expressed in glioma cells, showed an increasing gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Fig. 4D). In the non-tumor/healthy area, the number of TMEM119-positive cells is significantly higher than the number of positive cells present in the tumor center. Individual patient analysis showed that 10 (about 60%) out of 17 patients had an increase in the number of TMEM119-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 4E). Accordingly, TMEM119 gene expression is significant increase in the periphery and in the non-tumor area (Fig. 4F).

In addition, cumulative analysis for CD86, highly expressed in macrophage-microglia and also in B and T cells, showed an increasing gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Fig. 5D). In the periphery and in the non-tumor/healthy area, the number of CD86-positive cells is significantly higher than the number of positive cells present in the tumor center. Consistent analysis of individual patients showed that 15 (about 90%) out of 17 patients had an increase in the number of CD86-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 5E).

Similar results also for CD206, a membrane antigen expressed on the surface of immature dendritic cells and in phagocytic microglia. Cumulative analysis for CD206 showed an increasing gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Fig. 6D). In the periphery and in the non-tumor/healthy area, the number of CD206-positive cells is significantly higher than the number of positive cells present in the tumor center. Consistent analysis of individual patients showed that 13 (about 75%) out of 17 patients had an increase in the number of CD206-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 6E).

Similar results were obtained for ARG1 (a marker of M2-polarized microglia expressed in neutrophils, myeloid derived suppressor cells and neural stem cells) and iNOS (a pro-inflammatory marker of M1 microglia), however without reaching statistical significance. In fact, cumulative analysis for ARG1 and iNOS showed a tendency to increase gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Figs. 7D–8D). Analysis of individual patients showed that 7 (about 58%) out of 12 patients had an increase in the number of ARG1-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 7E). In 5 patients no ARG1 positive staining was shown. Concerning iNOS, analysis of individual patients showed that 7 (about 41%) out of 17 patients had an increase in the number iNOS-positive cells in the non-tumor/healthy area compared to the tumor core (Fig. 8E) and in 8 (about 47%) patients showed the same percentage in positive iNOS-staining.

Contrary to what has been described so far, CD204-pro-tumorigenic marker, expressed in microglia/macrophages and in dendritic cells, shows a descending gradient starting from the center of the tumor and moving toward the non-tumor/healthy area (Fig. 9D). In the non-tumor/healthy area, the number of CD204-positive cells is significantly lower than the number of positive cells present in the tumor center. Consistent analysis of individual patients showed that 14 (about 82%) out of 17

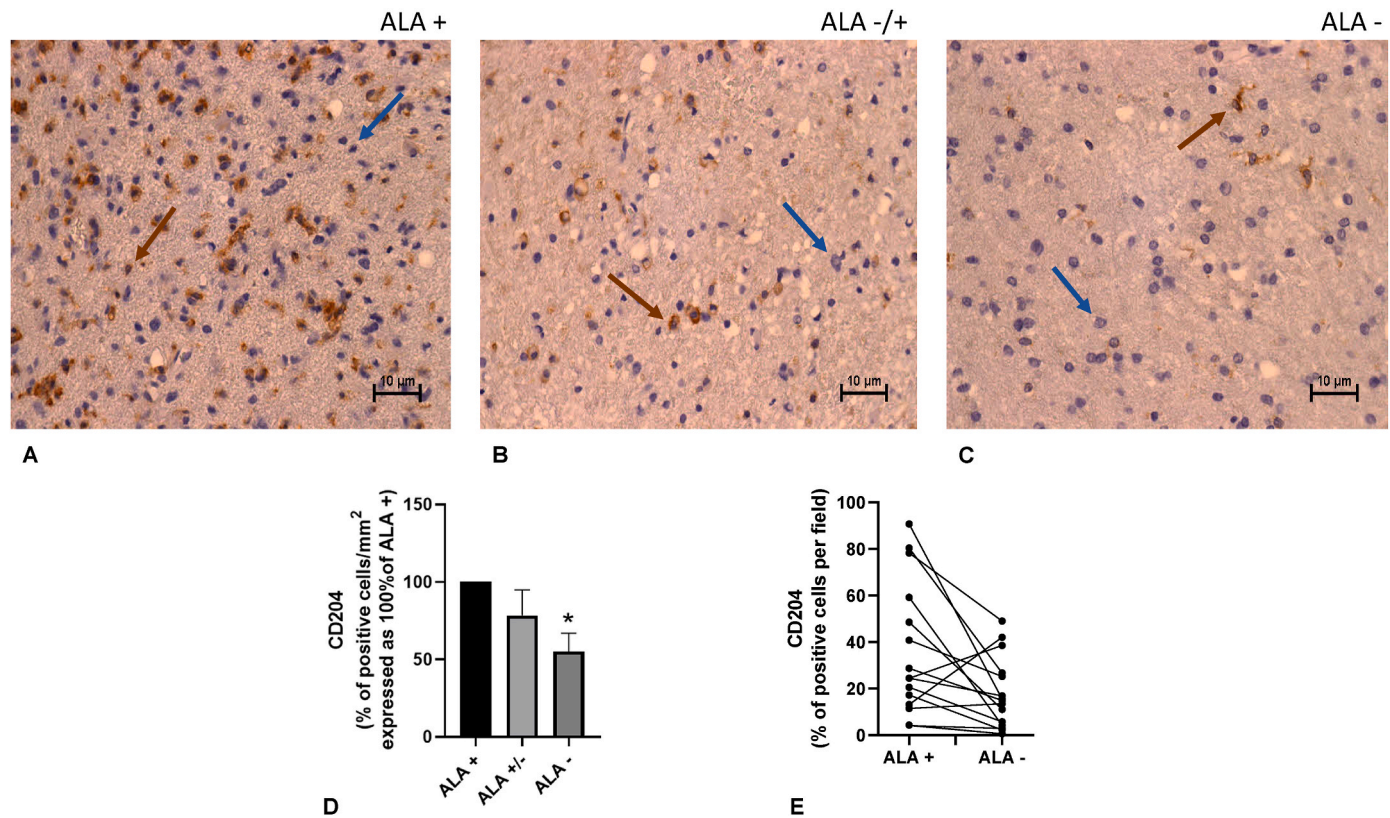


Fig. 9. CD204 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for CD204 (magnification: 20 ×). Brown arrows point to CD204 stained cells, whereas blue arrows point to CD204 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean ± SEM of samples collected from 17 patients. Tumor versus non-tumor/healthy area * $p < 0.05$.

patients had an increase in the number of CD204-positive cells in the tumor core compared to the non-tumor/healthy area (Fig. 9E).

Finally, two markers, CD163 and P2RY12, showed no significant differences among the 3 areas studied (Fig.s. 10D and 11D). Fourteen (about 80%) out of 17 patients present the same percentage of P2RY12 positive cells in the areas studied, while 9 (about 52%) out of 17 patients showed higher percentage of CD163 positive cells in the non-tumor/healthy area of the brain compared to the tumor center (Fig.s. 10E and 11E). CD163 is mainly expressed in microglia/macrophage cells but it is also present in glioma cells. P2RY12 is expressed in immune cells-

3. Discussion

Three different situations emerged from our study of the markers of microglial/macrophage-related and of other cell types in GBM patients: i) most of markers show an ascending gradient from the tumor center to the non-tumor/healthy area of the brain; ii) one marker shows a descending gradient, going from the center of the tumor to the non-tumor/healthy brain area; iii) two markers show no gradients.

In the first description we found: IBA1, TMEM119, CD206 and CD86 with significant differences, and iNOS and ARG1 with a clear trend but no statistical significance. In the second one we find CD204, while in the third situation we find CD163 and P2RY12.

Abundant presence of GAMs was observed not only in the tumor core (ALA + area) but also in areas relatively distant from it (ALA ± as well as ALA - areas). Two different GAM morphologies were observed in our

samples, with an amoeboid morphology prevalent in the areas closer to the tumor core and a branched morphology prevalent in areas distant from the tumor (Lier et al., 2021). As mentioned above, microglia constantly 'senses' the local environment through their multiple branches and highly motile processes in their resting state, under normal physiological conditions. These processes are in continuous motion, protruding and retracting to cover long distances and survey large brain areas. In the case of a brain injury or neuroinflammation, microglia become highly activated and gradually change from a ramified shape to an amoeboid form, that is associated with phagocytosis and proinflammatory function (Sasaki, 2017; Caponegro et al., 2021). Such ability to rapidly react has been described as "microglial activation", which will depend on the type of stimulus, time after stimulation and factors present in the local TME (Caponegro et al., 2021; Dello Russo et al., 2017; Zeiner et al., 2019).

Our data support the concept that an active 'recruitment system' exists for microglia and immune cells in the presence of tumor cells (Zhou et al., 2023; Wang et al., 2021). We were able to show that this phenomenon occurs in areas distant from the tumor core. The present immunohistochemistry data supports this observation. In fact, in the ALA - areas, which are located at a significant distance from the tumor core, IBA+, TMEM+, CD206+ and/or CD86+ cells represent a significant infiltration. Considering that the ALA + area is larger than the gadolinium enhancement at MRI and that the ALA pale (±) area extends well beyond the limit of the 'radiologically evident' tumor, the present observations give insight on the real extension of the inflammatory

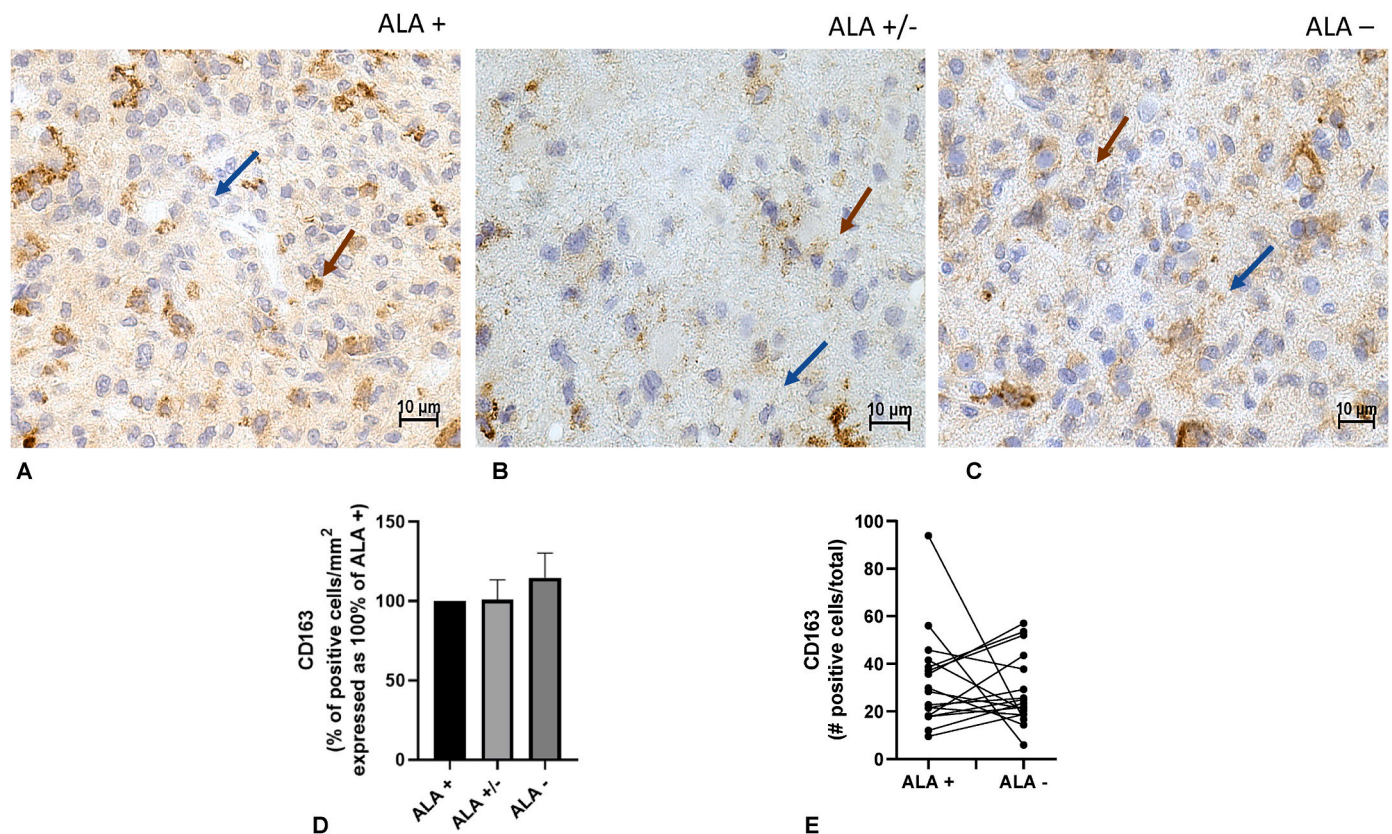


Fig. 10. CD163 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for CD163 (magnification: 40 ×). Brown arrows point to CD163 stained cells, whereas blue arrows point to CD163 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean ± SEM of samples collected from 17 patients.

recruitment phenomenon (Müther et al., 2019).

On this regard, a 5-ALA-based sampling appears to be superior to an imaging-based approach. MRI gadolinium enhancement area matches blood-brain barrier impairment, possibly underestimating the biological extent of the tumor burden (Kim et al., 2018). Indeed 5-ALA, being a biological rather than a permeability marker, has been shown to mark tumor tissue volume exceeding gadolinium enhancing tumor volume (Eatz et al., 2022). In other words, the extension of the ALA + area grossly corresponds to the Gadolinium enhancing area on MRI T1W images, whereas the ALA± and ALA - areas extend beyond the limits of the MRI enhancing tumor.

Based on these differences between ALA-5 and gadolinium marking features, our findings support the notion that GAM and in general immune cell infiltration starts already at the far periphery of the tumor, well beyond its margins.

In our study, we utilized targeted sampling within different 5-ALA fluorescent areas to examine the cell transition. Our observations revealed that microglia transitioned towards the pro-oncogenic/immunosuppressive M2 GAM phenotype. Notably, we observed the presence of CD 86 and CD206 + cells, which are markers of M2 polarization, in areas located far from the tumor core (ALA - and ALA +/-), supporting the hypothesis that an immunologically depressed area exists, which embraces the tumor well beyond its margins (Trevisi and Mangiola, 2023). This suggests that M2 polarization forms a "shell" around the tumor core, possibly sheltering from autoimmunity and

likely promoting immune escape.

With the limitation of a preliminary experience, this is the first study showing that a differential GAM and immune cell topographic distribution can maintain an immunologically downregulated environment.

Previous literature on the topic did not use the sampling technique as a systematic approach, thereby referring to a simplistic model (core vs periphery) without any validated methodology to define the tumor infiltrative margins. The observation that these phenomena already occur in the ALA-areas, therefore well beyond the tumor infiltrative edges (and hence, over the radiologically evident tumor) sheds light to the great extension of the immunosuppressive phenomenon.

This could represent the basis for a clearer evaluation of the 'spatiality of the immunomodulatory response', as it is explicitly this area, the far periphery beyond the evident tumor, that is not surgically resected and possibly poses the 'micro-environmental' bases of resistance to treatments, escape from autoimmunity and ultimately disease relapse.

4. Conclusions

Microenvironmental contribution seems therefore critical to gain a better understanding of the unique challenges GBM poses.

Admitting that the present study should be considered as preliminary, nonetheless we were able to clearly show that a microglia-macrophage and immune cell activation gradient exists, based on proximity to tumoral margins and degree of infiltration. This

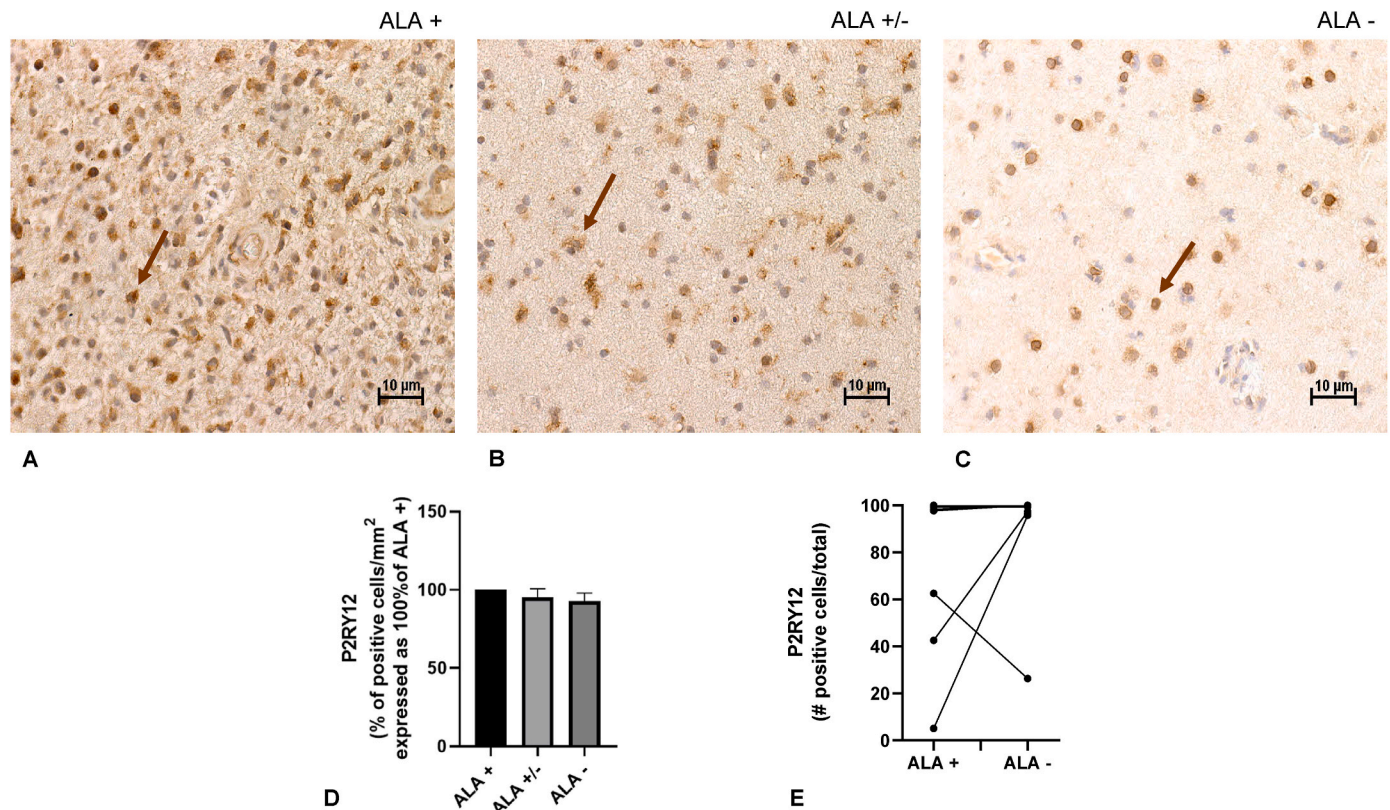


Fig. 11. P2RY12 staining in human GB specimens: the photographs show a representative tumor field (A), a representative periphery field (B) and a representative non-tumor/healthy area (C) stained for P2RY12 (magnification: 10 ×). Brown arrows point to P2RY12 stained cells, whereas blue arrows point to P2RY12 negative cells, respectively. The histograms represent the cumulative (D) and the individual (E) analysis. Data are expressed as mean ± SEM of samples collected from 17 patients.

corresponds to a modification of specific markers (CD86, CD204, CD206, ARG1 and iNOS) in areas far from the biologically active tumor, not displaying a clear neoplastic infiltration, and well beyond the radiologically evident enhancement.

These observations match with literature evidence supporting the idea that the magnitude of the diverted inflammation is a ‘extensive’ rather than a ‘local’ phenomenon, which could possibly play a role in disease resistance and relapse.

CRediT authorship contribution statement

Lucia Lisi: Writing – original draft, Formal analysis, Data curation. **Alessandro Olivi:** Supervision. **Gabriella Maria Pia Ciotti:** Methodology. **Salvatore Marino:** Investigation. **Chiara Ferraro:** Methodology. **Grazia Menna:** Investigation. **Maria Martire:** Visualization. **Giovanni Pennisi:** Investigation. **Pierluigi Navarra:** Writing – review & editing, Supervision. **Giuseppe Maria della Pepa:** Writing – original draft, Investigation, 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.

Data availability

Data will be made available on request.

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