

Microglia immunophenotyping in gliomas

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Abstract. Microglia, once assimilated to peripheral macrophages, in gliomas has long been discussed and currently it is hypothesized to play a pro-tumor role in tumor progression. Uncertain between M1 and M2 polarization, it exchanges signals with glioma cells to create an immunosuppressive micro-environment and stimulates cell proliferation and migration. Four antibodies are currently used for microglia/macrophage identification in tissues that exhibit different cell forms and cell localization. The aim of the present work was to describe the distribution of the different cell forms and to deduce their significance on the basis of what is known on their function from the literature. Normal resting microglia, reactive microglia, intermediate and bumpy forms and macrophage-like cells can be distinguished by Iba1, CD68, CD16 and CD163 and further categorized by CD11b, CD45, c-MAF and CD98. The number of microglia/macrophages strongly increased from normal cortex and white matter to infiltrating and solid tumors. The ramified microglia accumulated in infiltration areas of both high- and low-grade gliomas, when hypertrophy and hyperplasia occur. In solid tumors, intermediate and bumpy forms prevailed and

there is a large increase of macrophage-like cells in glioblastoma. The total number of microglia cells did not vary among the three grades of malignancy, but macrophage-like cells definitely prevailed in high-grade gliomas and frequently expressed CD45 and c-MAF. CD98⁺ cells were present. Microglia favors tumor progression, but many aspects suggest that the phagocytosing function is maintained. CD98⁺ cells can be the product of fusion, but also of phagocytosis. Microglia correlated with poorer survival in glioblastoma, when considering CD163⁺ cells, whereas it did not change prognosis in isocitrate dehydrogenase-mutant low grade gliomas.

Introduction

Microglia has been categorized as quiescent ramified microglia with small round cells and numerous branching processes, amoeboid or reactive microglia devoid of branching processes, macrophages and perivascular microglia (1). They can be intrinsic to the central nervous system (CNS) as resident microglia, or blood-borne from the monocytic phagocytosing system of the blood. Both derive from the yolk sac, the resident microglia earlier and later the cells destined to bone marrow for hematopoiesis and migrating to the CNS crossing the blood-brain barrier (BBB) in case of lesions.

In normal CNS, resident microglia is 5-10% of cells (2,3). Microglia/macrophages increase in low-grade gliomas (LGG) (4), especially in pilocytic astrocytomas (5), and progressively with the glioma grades (6), reaching 30% of cells in high grade gliomas (HGG), closely correlating with vascular density (7-11), developing around and inside the tumors and clustering around vessels and necroses.

Contrary to what is known from other CNS diseases and from microglia assimilation to peripheral macrophages, glioma-associated M/M (GAMs) are today prevalently interpreted as favoring glioma cell migration and growth (12-15). GAM polarization into the two functional profiles M1 (classically activated, pro-inflammatory) and M2 (alternatively activated, immunosuppressive) has long been discussed (12,13-19). Recently, GAMs have been shown to overlap only partial with the M1 and M2 phenotypes (20). Glioma cells would establish an immunosuppressive tumor environment, promoting GAM recruitment and proliferation, polarizing them toward the M2 phenotype and remaining unaffected by their phagocytosing and anti-tumorigenic functions (16,19-29). In this way they would be ‘friends’ and not ‘foes’ to gliomas (16,30). GAMs are recruited

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Abbreviations: BBB, blood-brain barrier; BC, bumpy cell; CD, cluster of differentiation; CNS, central nervous system; FFPE, formalin fixed, paraffin embedded; FRP, final reaction products; GAMs, glioma-associated microglia/macrophages; GB, glioblastoma; gDNA, genomic DNA; GICs, glioma initiating cells; H&E, haematoxylin and eosin; HGG, high grade gliomas; HIA, high infiltration area; HIER, heat-induced epitope retrieval; HPF, high power fields; Iba1, allograft inflammatory factor 1; IDH, isocitrate dehydrogenase; IF, intermediate form; IHC, immunohistochemistry; LGG, low-grade gliomas; M, macrophagic form; MIA, mild infiltration area; MM, microglia/macrophages; NOS, not otherwise specified; RA, regressive area; RM, reactive ramified microglia; ST, solid tumor; TLRs, Toll-like receptors; TPLSM, two-photon laser scanning microscopy; WHO, World Health Organization

Key words: microglia, macrophages, gliomas, immunosuppression, phagocytosis

by glioma cells through chemoattractant factors and, in turn, they stimulate tumor growth (26,31,32). Both glioma cells and GAMs promote angiogenesis (33,34).

In tissue, four antibodies are currently used to demonstrate GAMs, i.e., allograft inflammatory factor 1 (Iba1), CD16, CD68, and CD163. They reveal different antigens with different final reaction products (FRP) and with different localization in the cells: In cytoplasm for Iba1, in lysosomes for CD68 and on cell membranes for CD16 and CD163. Various GAM forms can be demonstrated, going from ramified microglia to granular macrophage-like cells, which correspond to different antigen expression. Given the uncertainties still existing on the significance of GAMs, one wonders to which stimuli so various cell forms and immunohistochemical behaviors respond in the different tumor districts. The aim of this work is to clarify their functional significance after the systematic study of a broad series of human gliomas and to interpret them on the basis of the information available on their functions.

Materials and methods

Brain tumor specimens. The study has been carried out on 98 adult glioma specimens from our archive and operated on at different Neurosurgery Units of the Piedmont Region and at the Istituto Neurologico 'Carlo Besta' IRCCS Foundation, Milan, Italy. The series was composed of 3 pilocytic astrocytomas, 20 diffuse astrocytomas (16 isocitrate dehydrogenase (IDH)-mutant and 4 IDH-wild-type), 4 anaplastic astrocytomas (1 IDH-mutant and 3 IDH-wild-type), 26 oligodendrogliomas (20 IDH-mutant and 1p/19q-codeleted and 6 not otherwise specified (NOS)), 10 anaplastic oligodendrogliomas (6 IDH-mutant and 1p/19q-codeleted and 4 NOS) and 35 glioblastomas (GBs), IDH-wild-type. The histological diagnosis was made according to the current World Health Organization (WHO) guidelines (35). As controls, 10 tumor-free tissue samples were used. Surgical tumor samples were formalin fixed, paraffin embedded (FFPE) and cut in 5 μ m-thick sections. The study was in compliance with the local institutional review board and Committee on Human Research and with the ethical human subject principles of the World Medical Association Declaration of Helsinki Research. Written informed consent of patients was obtained after the Ethics Committee approval.

Immunohistochemistry (IHC). Beside haematoxylin and eosin (H&E) staining, immunohistochemical analyses were performed using a Ventana Full BenchMark[®] XT automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) and the UltraView[™] Universal DAB Detection Kit (Ventana Medical Systems Inc.) as detection system. Heat-induced epitope retrieval (HIER) was performed in Tris-EDTA, pH 8. Primary antibodies are listed in Table I. Double immunostainings for c-MAF/CD68, c-MAF/CD163, Iba1/CD34 and CD68/CD34 were performed with the ultra-view[™] Universal Alkaline Phosphatase Red Detection Kit (Ventana). Negative controls were obtained by omitting the primary antibodies. Observations were made on a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam5 MR5c and coupled to an Imaging system (AxioVision Release 4.5; Carl Zeiss).

Molecular genetics. Genomic DNA (gDNA) from FFPE tumor samples was isolated using the QIAamp DNA Mini kit (Qiagen NV, Venlo, The Netherlands). Search for somatic point mutations in *IDH1* Arg132 (exon 4) (GenBank sequence NM_005896) and *IDH2* Arg172 (exon 4) (GenBank sequence NM_002168) hot-spot codons was performed by Sanger direct sequencing on an ABI[®] 3130 Genetic Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as published (36). The 1p/19q chromosomal status was assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA-MLPA Kit P088-B2 (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions. Fragment analysis was performed on an ABI[®] 3130 Genetic Analyzer (Thermo Fisher Scientific, Inc.).

GAM frequency and survival analysis. The frequency of GAMs was quantified by counting the number of immunopositive cells for each antibody and for each cell form (see Results) in five randomly selected microscopic high power fields (HPF) at x400 magnification per section and by calculating the mean values. Only cells were counted the nucleus of which was visible in counterstained sections. The number of GAMs was compared with the three grades (II-IV) of malignancy. CD163 immunohistochemical expression was evaluated according to the average frequency of positive cells (<10, 20-50, 50-100, >100) and distribution (perivascular or scattered in the parenchyma) after examining 5 randomly selected fields *per* tumor section at x400 high-power magnification, with a semi-quantitative scoring system including four categories: 0, 1, 2 and 3 (Table II) and compared with the overall survival (OS) of patients. OS was defined as the time from date of diagnosis until the time of death or last follow-up of the patient. The comparison was made also by scoring the CD163 staining in two categories of expression: Low (including the above 0 and 1 categories) and high (including the above 2 and 3 categories) (Table II). The correlation between CD163 expression and OS was investigated in the tumor series also after stratification for IDH1/2 mutation status.

Statistical analysis. The correlation between tumor grade and immunohistochemical expression of CD68 or CD163 was analyzed by investigating differences among the groups with a one-way ANOVA and Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. Survival curves were estimated using the Kaplan-Meier method and comparison between them were performed by the log-rank test (Mantel-Cox). Data were analyzed with the SPSS v23.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Immunohistochemical analysis. Four groups of GAM forms were identified immunohistochemically, that differed not only for positive or negative staining, but also for staining intensity: i) normal ramified microglia with a small elongated nucleus and polar processes with a radial or longitudinal branching; ii) reactive microglia (RM); iii) cells with a roundish and granular macrophagic form (M); and iv) cells of intermediate form (IF), with very thick and short processes, or simply roundish

Table I. List of primary antibodies used for immunohistochemistry.

Antibody (Clone)	Source	Dilution	Code	Manufacturer
Ki-67 (MIB-1)	Mouse	1:100	M7240	Dako
GFAP	Mouse	1:200	M0761	Dako
CD34	Mouse	Pre-diluted	790-2927	Ventana
Iba1	Rabbit	1:500	019-19741	Wako Chemicals
CD68 (KP-1)	Mouse	Pre-diluted	790-2931	Ventana
CD16 (SP175)	Rabbit	Pre-diluted	760-4863	Ventana
CD163(MRQ-26)	Mouse	Pre-diluted	760-4437	Ventana
c-MAF (M-153)	Rabbit	1:50	sc-7866	Santa Cruz Biotech.
CD45 (LCA)	Mouse	Pre-diluted	760-2505	Ventana
CD11b	Rabbit	1:100	AB52478	Abcam
CD98	Rabbit	1:100	AB108300	Abcam

Table II. Four and two-score system for evaluation of CD163 immunostaining.

Score	Frequency of positive cells (per x400 HPF)	Distribution
0 ^a	<10	Only perivascular
1 ^a	20-50	Perivascular and scattered in the parenchyma
2 ^b	50-100	Perivascular and scattered in the parenchyma
3 ^b	>100	Perivascular and scattered in the parenchyma

^aLow, ^bhigh.

bumpy cell (BC) forms. The first two cell types stained prevalently with Iba1 and CD16, the third group with CD68 and CD163 and the last group showed a variable staining with the 4 antibodies.

Normal cortex. Iba1 stained diffusely 10-15 cells per x400 HPF with thin processes and the typical branching (Fig. 1A). CD68 revealed 7-8 cells per x400 HPF with a FRP of fine granules in the cytoplasm and/or short processes. CD16 showed the same finding as Iba1. Ischemic neurons showed CD16-positive nuclei with a round or elongated form. CD163 was almost negative.

Gliomas. Gliomas are heterogeneous tumors, therefore, selected areas with defined cell distribution patterns were chosen from all the cases for comparison, corresponding to mild (MIA) and high (HIA) tumor infiltration areas, solid tumor (ST) and regressive areas (RA). In LGG form types 2 and 4 and in HGG types 3 and 4 prevailed. In ST and RA of HGG most part of GAMs were, therefore, positive for CD68 and CD163.

Diffuse astrocytomas. Iba1. In MIA, cells appeared as elegant RM with thin processes, longer than in normal cortex, in number of 20-25 positive cells per x400 HPF (Fig. 1B). In

HIA, they increased progressively with the increase of tumor cell number until to 100-200 cells per x400 HPF (Fig. 1C). In ST the number of positive cells decreased to 80-120 cells per x400 HPF and IF and M phenotypes with and without processes prevailed, not only around vessels, but also scattered in the tissue.

CD68. In MIA, RM cells with positive granules in the cytoplasm or short processes occurred in number of 15-20 per x400 HPF. In HIA, the positive cells increased to 50-100 per x400 HPF with coarser granules and broader short branching. They assumed the IF or M aspects approaching ST. In the latter, the density of positive cells decreased to 40-50 per x400 HPF, assuming prevalently the roundish form of M. Perivascular M clusters could be observed with a frequency depending on the vessel density as well as rare scattered forms. Some IF and most M forms stained more intensely than with Iba1.

CD16. The distribution was similar to that of Iba1 (Fig. 1D). Ischemic neurons entrapped in the tumor appeared as ball-like positive figures.

CD163. Positive cells with a M-like or with an IF or BC appearance were rare and mostly with an intraluminal or perivascular localization; positive cells scattered in ST were even rarer. CD163 was positive only in a quota of CD68+ cells.

Oligodendrogliomas. The tumor growth could be diffuse or nodular. In the first type, GAMs were diffusely increased in number, whereas in the second one GAMs were limited to a thin strip immediately close to the tumor and in ST they were less numerous and mainly IFs or BCs. The findings were similar to those of diffuse astrocytomas, but with a great difference between MIA and HIA: 30-40 vs. > 200 positive cells. In ST (nodular growth), IFs and M forms (Fig. 1E) prevailed on RM phenotype (Fig. 1F), both in perivascular position and scattered in the tissue. CD16 and CD163 behaved as in diffuse astrocytomas. In MIA and HIA most CD163+ cells are perivascular (Fig. 1G). In the same areas, Iba1+ RM did not crowd around small vessels (Fig. 1H).

Anaplastic astrocytomas and oligodendrogliomas. The only difference was that in astrocytomas there was no increase of vessels and, therefore, the number of perivascular M cells did not vary; in anaplastic oligodendrogliomas the vessels increased

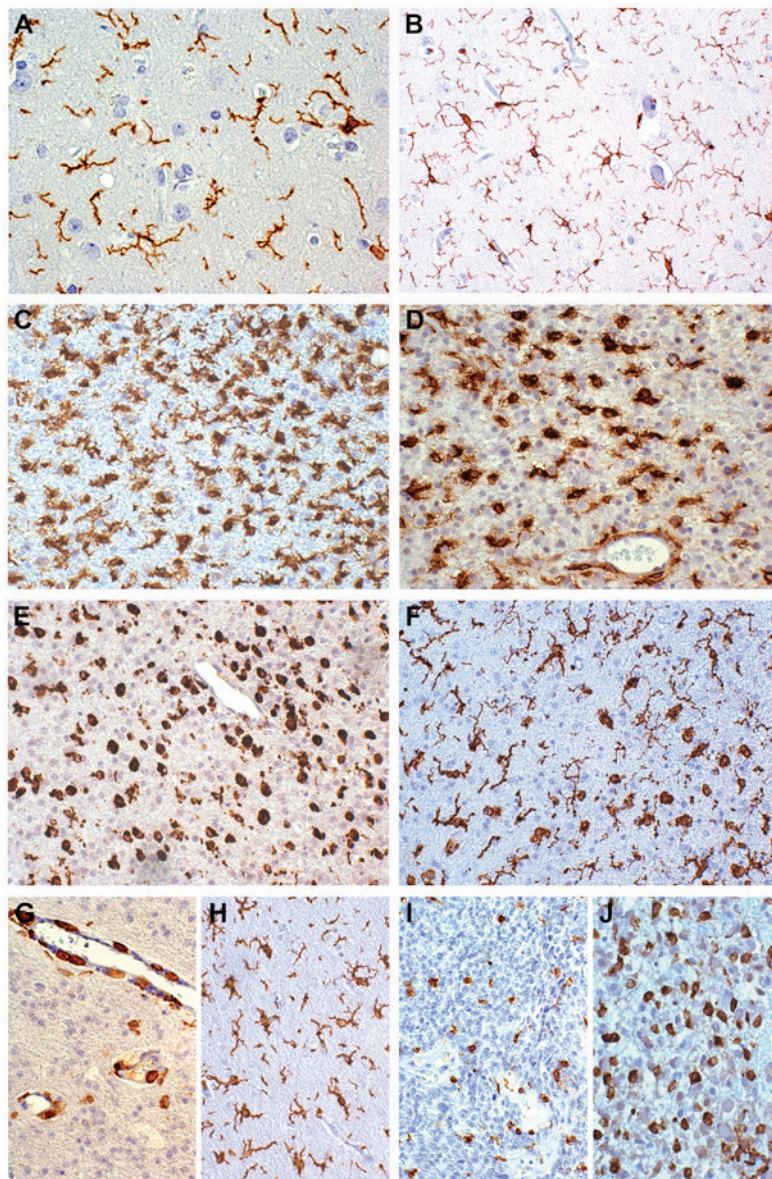


Figure 1. Immunohistochemical characterization of microglia/macrophages. (A) Resident ramified microglia in the normal cortex, Iba1 staining, x400. (B) Diffuse astrocytoma. Reactive ramified microglia in mild tumor infiltration, Iba1 staining, x200. (C) Diffuse astrocytoma. Reactive ramified microglia in high tumor infiltration, Iba1 staining, x200. (D) Diffuse astrocytoma. Intermediate and macrophagic forms, CD16 staining, x200. (E) Oligodendroglioma. Cells with a macrophage-like aspect in solid tumor, CD68 staining, x200. (F) Oligodendroglioma. Reactive ramified microglia and macrophagic forms, Iba1 staining, x200. (G) Oligodendroglioma. CD163⁺ cells were mainly found around vessels, CD163 staining, x200. (H) Oligodendroglioma. In MIA and HIA Iba1⁺ RM did not crowd around small vessels, Iba1 staining, x200. (I) Anaplastic oligodendroglioma. Intermediate and macrophagic forms in solid tumor, Iba1 staining, x200. (J) Anaplastic astrocytoma. Intermediate and macrophagic forms in solid tumor, CD163 staining, x200. All stained with DAB. Iba1, allograft inflammatory factor 1; DAB, 3,3'-diaminobenzidine; CD, cluster of differentiation; MIA, mild infiltration area; HIA, high infiltration area; RM, reactive ramified microglia.

as well as microvascular proliferations and M forms and IFs increased correspondingly (Fig. 1I). In grade III gliomas an increase of CD163-positive cells was observed (Fig. 1J).

Pilocytic astrocytomas. GAMs showed the same frequency as in diffuse and anaplastic astrocytomas with a prevalence of M forms and IFs.

Glioblastomas. In MIA and HIA the findings were similar to those described in II and III grade gliomas. In ST the number of RM cells strongly reduced and Iba1⁺/CD16⁺ IFs and mainly M forms increased (Fig. 2A), especially in perinecrotic or perivascular clusters as CD163⁺ cells (Fig. 2B). Iba1⁺ M forms can show a density >200 cells per x400 HPF, representing >40% of tumor cells (Fig. 2C). In the transition from ST to RA, M-like forms

increased. They stained more intensely with CD68 and CD163 (Fig. 2D) than with Iba1 and CD16. In necroses larger than the microscopic field at x400 HPF, almost all cells could be represented by M forms reaching, therefore, the 100% of cells. IFs stained more intensely with Iba1 and CD16 whereas M forms with CD68 and CD163. GAMs crowded in glomerular structures (Fig. 2E). M cells showed in variable number a nuclear positivity for c-MAF, and this was well evident in c-MAF/CD163 (Fig. 2F) and c-MAF/CD68 double immunostainings.

CD11b and CD45. The former was weakly expressed in a quota of GAMs, whereas the latter was positive in a quota of CD68⁺ and CD163⁺ cells. Roughly CD45⁺ cells corresponded to CD163⁺ cells (Fig. 2G, H).

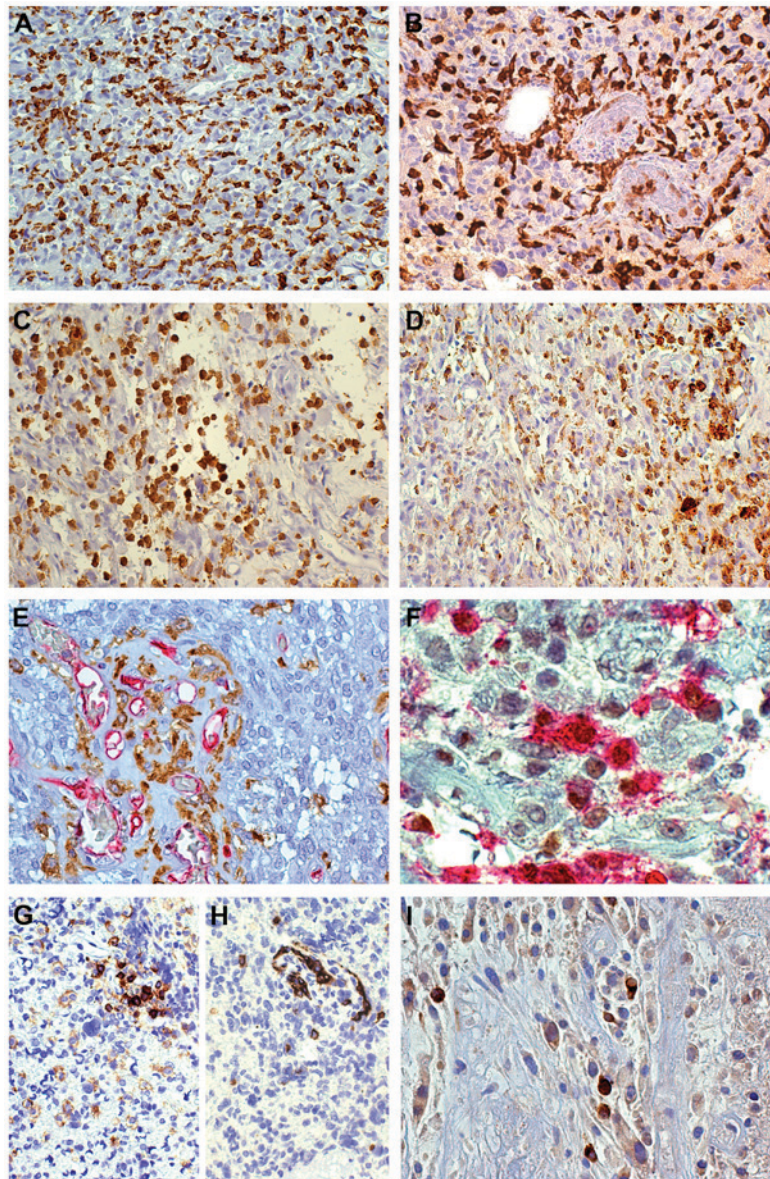


Figure 2. Immunohistochemical characterization of microglia/macrophages. (A) GB; High frequency of CD16⁺ intermediate forms in solid tumor, CD16 staining, x200, DAB. (B) GB; Perivascular CD163⁺ macrophagic forms, CD163 staining, x200, DAB. (C) GB; Iba1⁺ macrophagic forms, Iba1 staining, x200, DAB. (D) GB; Passage from a solid tumor to a regressive area: CD163⁺ cells are rare in the former and strongly positive and frequent as macrophagic forms in the latter, CD163 staining, x200, DAB. (E) GB; Crowding of GAMs in glomerular structures, Iba1 (DAB) and CD34 (RED) stainings, x200. (F) GB; Macrophagic forms with c-MAF⁺ nuclei and CD163⁺ cytoplasm, c-MAF (DAB) and CD163 (RED) stainings, x630. (G) GB; Perivascular CD45⁺ M forms, CD45 staining, x200, DAB. (H) GB; Perivascular CD163⁺ M forms, CD163 staining, x200, DAB. (I) GB; CD98⁺ macrophage-like cells, CD98 staining, x400, DAB. CD, cluster of differentiation; DAB, 3,3'-diaminobenzidine; HPF, high power fields; GB, Glioblastoma; Iba1, allograft inflammatory factor 1; GAMs, glioma-associated microglia/macrophages.

In some GBs, CD98 was co-expressed with GFAP in scattered cells in ST areas (Fig. 2I).

Correlation of GAMs with histological grade and with survival. Iba1⁺ and CD16⁺ cells prevailed in LGG, whereas CD68⁺ and CD163⁺ cells prevailed in HGG. If all GAM forms (RM, BC, IF and M), positive to one of the 4 antibodies were considered, there was no correlation with the histologic grade, because the high number of RM in LGG paralleled that of M forms in HGG. Iba1⁺ cells did not show a significant difference in number among WHO II-IV grade gliomas, nor CD16⁺ cells. On the contrary, expression levels of CD68⁺ and especially CD163⁺ cells increased with malignancy grade with a statistically significant difference between II and IV grade (Fig. 3).

CD163-positive BC and M were definitely in a higher number in HGG than in LGG.

As for survival analysis, considering the whole series of gliomas (WHO grade II-IV), CD163 immunohistochemical expression significantly correlated with a shorter OS for patients, both applying the 4-score system (log-rank test, $P=0.0001$) and using a 2-score system (log-rank test, $P=0.0001$) for CD163 (Fig. 4A and B).

Evaluating the correlation between OS and CD163 within each single tumor type and grade, no statistically significant correlation was found. Also considering the group of II grade tumors (astrocytomas and oligodendrogliomas), no correlation emerged, whereas a significant correlation was found in the group of III grade gliomas, astrocytomas + oligodendrogliomas

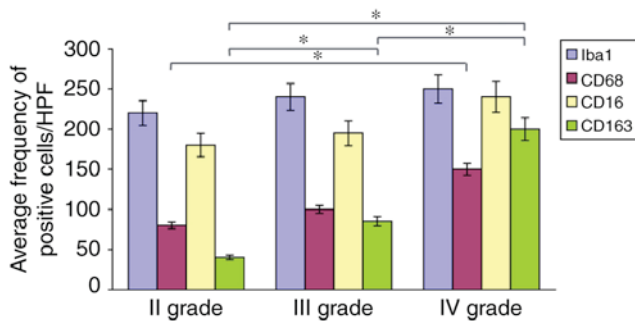


Figure 3. Correlation of the microglia/macrophages markers with the histological grade in 95 patients with WHO II-IV grade gliomas. The number of MM immuno-positive for CD68 and CD163 correlated with the grade of malignancy. Data are mean values \pm SE. * $P < 0.05$, one-way ANOVA and Tukey's test. CD, cluster of differentiation; Iba1, allograft inflammatory factor 1; HPF, high power fields.

(log-rank test, $P = 0.028$) (Fig. 5A). Considering separately astrocytomas (II and III grade) and oligodendrogliomas (II and III grade), a significant correlation came out (log-rank test, $P = 0.007$ and $P = 0.008$, respectively) (curves not shown). These findings suggested that CD163, evaluated on the whole series of grade II-IV gliomas, has a prognostic value, however, completely absorbed by that of histological grade.

After stratification of patients for IDH1/2 mutation status, no significant correlation between OS and CD163 was found within the LGG series, whereas including III grade tumors a significant correlation was observed, but it reflected that of the histological grade (log-rank test, $P = 0.001$) (Fig. 5B). It is to be remarked that in our series IDH-wild-type II and III grade tumors were very few.

Microglia relationship with the tumor vasculature. In MIA and HIA of LGG, where only in oligodendrogliomas an increase of small vessels occurred in comparison with normal tissue, RM forms tremendously increased, without crowding on single vessels, but showing a high frequency in areas rich in vessels. On the contrary, adherence of single microglia cells to capillaries was frequent. In HGG, GAMs accumulated around vessels either as M forms or IFs and BCs or as unidentifiable forms, because secondary to pathoplastic influences, such as those of cells stretched around vessels.

Discussion

The greatest information about microglia and GAMs derives from studies on myeloid cells in tissues, *in vitro* cultures, in animal experimentation and recently from the Two-photon laser scanning microscopy (TPLSM) (37-39). The GAM types identified by the four relevant antibodies, more or less correspond to those described in GL-261 glioma model by TPLSM (38). In infiltration areas, there was no doubt on the origin of RM from resident microglia (27), more intensely stained with Iba1 and CD16 than with CD68 and CD163 and almost no macrophage was present (33).

The transition from HIA to ST was marked by an almost disappearance of RM forms, that could be reduced, destroyed or switched to serve a different function. In ST, IFs with thick short processes or BCs and round M forms without processes

were found, but in a less number than RM of infiltration areas, with the exception of their clustering around necroses or in perivascular position. They stained more intensely with CD68 and CD163. A quota of M forms were CD45^{high} (11,19,40) and stained with c-MAF (41) and roughly corresponded to CD163⁺ cells. They correspond, therefore, to blood-borne macrophages, unless resident microglia upregulates CD45 becoming thus responsible for most macrophages in gliomas (27). IFs and BCs can originate either from resident microglia or represent blood-borne macrophages, except that they correspond to the phagocytosing cells described by TPLSM (38). In HGG, CD68⁺ and CD163⁺ GAMs with M-like morphology prevailed. Contrary to the impression that they represent an immune or cytotoxic defense against the tumor, a pro-tumor function has been attributed to these cells, recruited and stimulated to proliferate by the glioma cells through TGF- β , prostaglandin E₂, CCL2, CX3CL1 and colony stimulating factor 1 (CSF-1) (23,26,42). Where the boundary between tumor and normal tissue was sharp, RM cells were limited to a narrow peritumor strip and this supports the idea that migrating neoplastic cells are the main motive of RM proliferation.

In HGG, despite GAMs show complement (CR1, CR3, CR4) and Fc- γ receptors, their phagocytic capacity is considered reduced (23), based on the relief that CR3⁺ microglia cells were never observed to kill glioma cells (43), but correlated with tumor proliferation rate (44). GAMs don't express cytokines for tumoricidal activity (21); in contrast, glioma-released factors induce in microglia cells, via Toll-like receptors (TLRs), the expression of membrane type 1 matrix metalloprotease (MT1-MMP) that promotes glioma expansion (12,45).

From a neuropathological point of view, one wonders what else CD68⁺ and CD163⁺ perinecrotic and perivascular cells could be if not cells phagocytosing tumor debris in regressive areas. The same could be true for macrophagic forms scattered in tumor proliferating areas. Here apoptosis occurs correlating with mitoses (46) and its decomposition products are known to be phagocytosed in few hours by macrophages (47).

M2 polarized cells are deduced to represent the greatest part of GAMs, independently of their phenotypes, including the rare CD163⁺ M forms of LGG and the tremendous amount of them in HGG, where it could be secondary to the BBB breakdown. Were it not for the increase of this cell component in GB, it could not be said that GAMs increase in HHG in comparison with LGG, for the great richness of the latter in RM forms. It was observed that M2 GAMs stain by CD163 (48-51), or express c-MAF (41), not exclusively, but prevalently. We confirm this findings, but, at the same time, we remark that the number of CD11b⁺ cells was too low, so that it could indicate only a quota of cells originating from resident microglia.

The relationship between vessels and GAMs is controversial. They increased near vessels or in infiltration areas in LGG and cluster in perivascular spaces in HGG, but, even if showing direct contacts with capillaries in both tumor types, they did not crowd around the small vessels in infiltration areas. In LGG, the finding of intra- and perivascular GAMs depended on the vessel number, that was higher in oligodendroglioma than in astrocytoma and, together with GAMs scattered in the parenchyma, prevailed in solid tumor upon its periphery. It has been reported that VEGF produced by glioma cells has a chemotactic effect on them, inducing their migration and

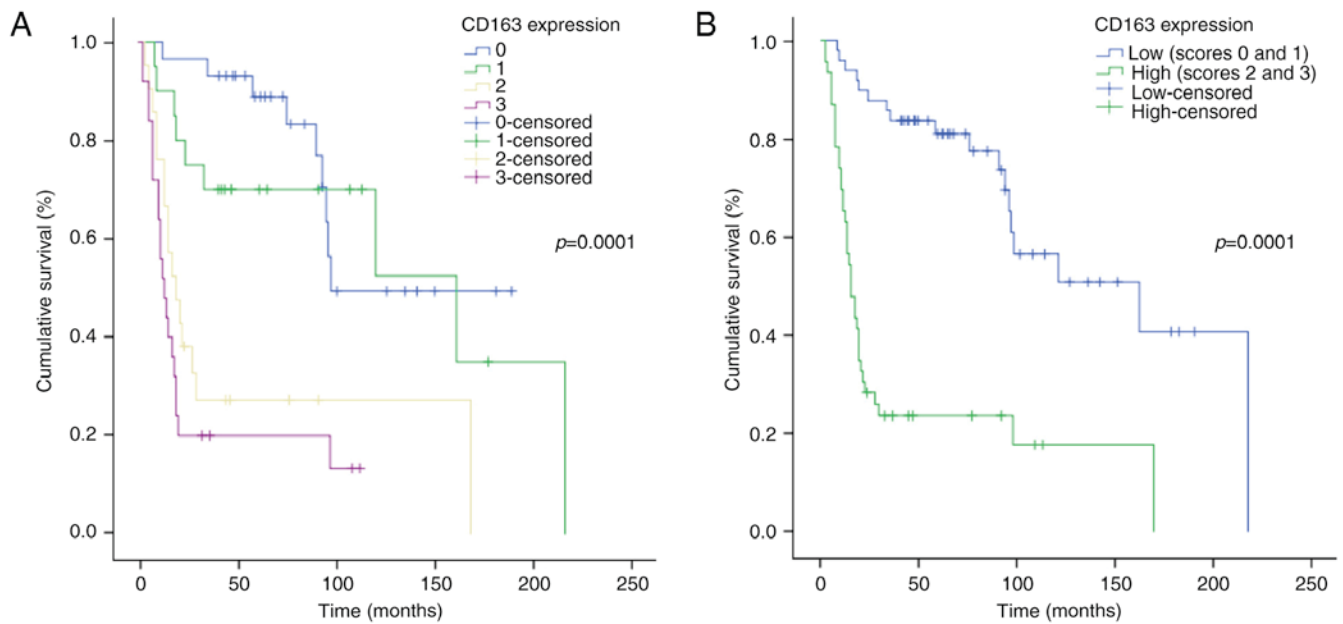


Figure 4. Kaplan-Meier survival analysis in 95 patients of WHO II-IV grade gliomas, according to the CD163 immunohistochemical expression. (A) Correlation of OS with CD163 levels according to a four-category (0, 1, 2, 3) scoring system. (B) Correlation of OS with CD163 levels according to a two-category (low and high) scoring system. The log-rank test was performed to determine statistical significance, with $P < 0.05$ considered as significant. OS, overall survival; CD, cluster of differentiation; WHO, World Health Organization.

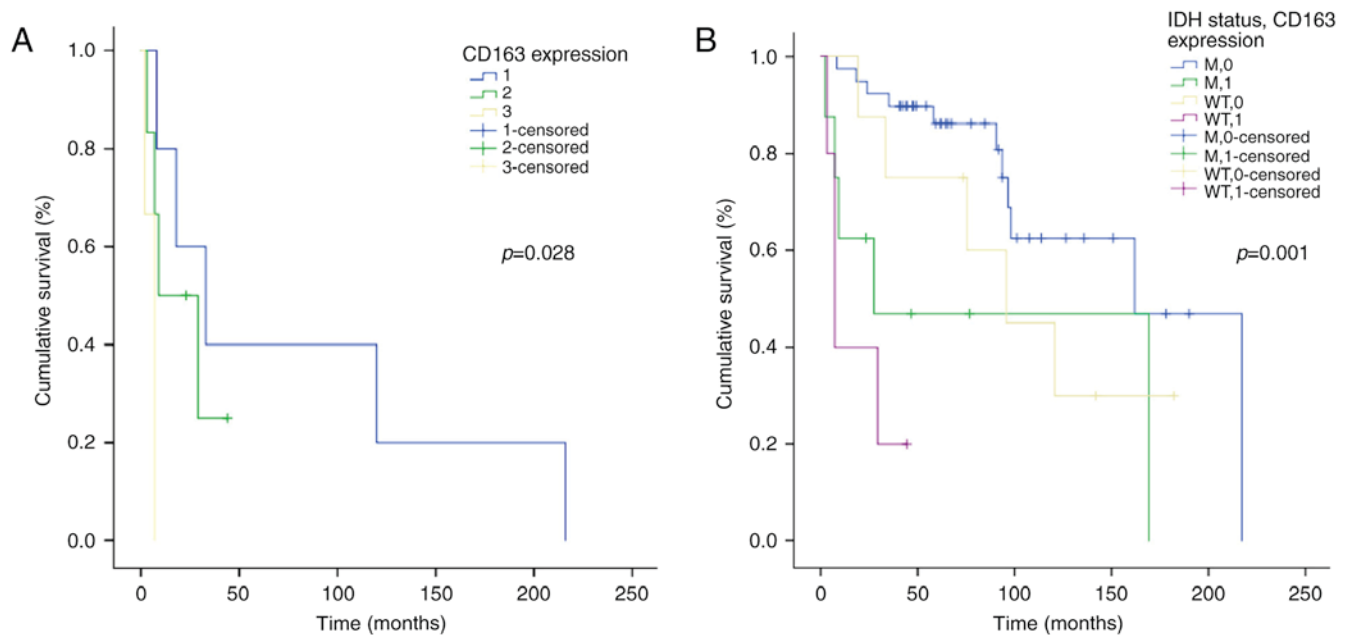


Figure 5. Kaplan-Meier survival analysis according to the CD163 immunohistochemical expression. (A) Correlation of overall survival (OS) with CD163 levels according to a four-category (0, 1, 2, 3) scoring system in WHO III grade glioma patients. (B) Correlation of OS with the IDH gene status and the CD163 expression (0=low, 1=high) in 60 patients of WHO II-III grade gliomas. The log-rank test was performed to determine statistical significance, and $P < 0.05$ was considered significant. CD, cluster of differentiation; M, mutated; WT, wildtype; WHO, World Health Organization; IDH, isocitrate dehydrogenase.

proliferation *in vitro* (52) and *in vivo* (53). GAMs are attracted by vessels and, in turn, promote angiogenesis (54,55). It must be remarked that perivascular IFs and M forms are mainly CD163+.

Despite the literature considers CD16 as indicating M1 (56) and CD163 M2 (31,48-50,57-59) polarization profile, IFs and BCs, independently of their origin from resident microglia or blood-borne macrophages, are not strictly recognizable as M1

or M2 polarized. It has been rather hypothesized a continuum M1-M2 functional state with the possibility that, upon different stimulations (60), both or neither one is expressed by GAMs (18-20,26). It must be considered that microglia cells undergo rapid changes (38), passing cell forms quickly into one another under the influence of the microenvironment; moreover, in compact tumor areas they can be mechanically modified.

The tumorigenic function of GAMs is supported *in vitro* by the interaction with glioblastoma stem cells (GSCs), that induce in them an immunosuppressive phenotype (31); GAMs, in turn, enhance the invasiveness of the glioma initiating cells (GICs) (32). In GB, an indirect proof is the correlation of GAMs not only with Ki-67/MIB-1, but also with the stem cell markers Nestin, SOX2 and CD133 (61). Recently, much attention is given to the relationship between GAMs, GSCs and vessels, through CCL2/CCR2, CXCL12/CXCR4, CX3CL1/CX3CR1, CSF-1 and periostin signalings; however, our knowledge on the relations between microglia, tumor cells and other components cannot be considered exhaustive (26).

Finally, in GB, it has been hypothesized a fusion between tumor cells and macrophages, denounced by CD98 positivity (62). This would be in line with the phagocytic capacity maintained in glioma cell lines and fetal rat normal glia (63,64). In our GBs, one wonders whether CD98⁺ cells are nothing else than macrophages that phagocytosed tumor cells, as it happens in non-neoplastic conditions, for example, where GFAP and myelin debris can be found in macrophages.

The inter-grade analysis didn't show a correlation of GAMs with survival if all the microglia forms demonstrated by the four antibodies were considered, because in LGG RM forms can reach the same frequency as M in HGG (4). The correlation with the tumor grade and with survival came out if only CD68⁺ and CD163⁺ M forms were considered. In GB, however, CD163⁺ M forms could not be directly related to malignancy, because they are associated to tumor structures that are typical of the malignant phenotype. The occurrence of CD163⁺ M forms in perivascular position should mean that they derived from the peripheral blood, because of the BBB disruption, which already by itself points out malignancy. CD163⁺ M or IF forms may have different interpretations; it is known that they increase from II to IV tumor grade. Considering together II grade astrocytomas and oligodendrogliomas, no correlation of CD163 expression with OS was found, whereas, in the group of III grade gliomas, a correlation was evident ($P=0.028$). Also putting together II and III grade astrocytomas or II and III grade oligodendrogliomas, a significant correlation was found. This is quite comprehensible in oligodendrogliomas, where an increase of vessels occurs, but not in astrocytomas, where the number of vessels does not increase. IDH status does not seem to have any correlation with GAMs in II grade gliomas; considering together II and III grade tumors a correlation came out, but also in this case it can be attributed to the short survival of anaplastic oligodendrogliomas.

It must be remarked that the number of CD163⁺ cells was higher than that of CD45⁺ cells; this means that M2 polarization could be independent of the origin of microglia.

Today most observations on GAMs demonstrate their favouring function on tumor progression. This conclusion is mainly derived from experiments on animal models and *in vitro* cultures. In the neuropathological practice, however, uncertainty still exists and the evaluation of GAMs must be given directly on tumor tissue and be part of the prognosis/diagnosis. The answer to the question if GAMs are 'friends' or 'foes' cannot be so sharp as in experimental condition, also because the increase of CD163⁺ forms might be secondary and not primary to malignancy.

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