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Original article

# A reappraisal of macrophage polarization in glioblastoma: Histopathological and immunohistochemical findings and review of the literature

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ABSTRACT

The survival rate in glioblastoma multiforme patients has scarcely improved in the last decades; however, many new therapeutic strategies have been theorized or developed for these neoplasias. Recently, the inverse correlation observed between patient prognosis and tumor-associated macrophages (TAMs) density in solid tumors has encouraged the development of anti-tumor strategies aiming to target TAMs. As expected, TAMs polarization is influenced by both macrophage localization and tumor microenvironment signals, resulting in a more complex scenario than the simple M1/M2 activation status. Macrophage polarization in glioblastoma has not yet been fully elucidated, and most results have been obtained in experimental non-human settings, with some apparent contradiction. The authors performed a histopathological and immunohistochemical study of 37 cases of glioblastoma in order to characterize the M1 and M2 macrophage populations within TAMs. A high prevalence of CD163+ M2-polarized macrophages was detected in this cohort, whereas iNOS+ macrophages were rarely found. The down-regulation of CD68 expression in microglia/macrophage infiltrating glioblastomas is also reported for the first time. Such a finding is associated with a specific location of TAMs within the lesion, as confirmed by the fact that CD68 staining was lower than CD163, mainly in perivascular areas.

The authors discuss the recent literature about the global scenario of macrophage plasticity and polarization in glioblastoma, and suggest some pivotal points for therapeutic applications.



## 1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive diffuse glioma of astrocytic lineage (WHO, grade IV (4th ed., 2007)). GBMs arise either from the progression of low grade glioma or rapidly in a *de novo* fashion. It has been reported in brainstem and cerebellum, but it is more common in the cerebral hemispheres and in age group 60–69 [1]. The prognosis of GBM is poor, with median overall survival of approximately 15 months and 8 months for newly diagnosed and recurrent disease, respectively [2,3]. Treatments are limited to microsurgical resection, followed in some cases, by chemotherapy and radiotherapy. In contrast to many other types of cancer, the survival rate has not changed significantly in the last decades. Indeed, the large amount of preclinical research results has not been translated into clinical benefit for patients, with a consequent sense of frustration [4]; however, all the research conducted in this field has contributed to a better understanding of disease progression.

From the first discovery of tumor-associated macrophages (TAMs) [5], a large number of experiments have been carried out in order to clarify the mechanisms of action and the role of such cells in neoplasia. To date, few papers have described activation of a specific macrophage phenotype (such as M1 and M2) in human malignant brain tumors.

Recently, anticancer immunotherapies focusing on macrophage ablation or repolarization have been proposed [6]. Moreover, the results concerning this specific research field are heterogeneous, and morphological-molecular correlations are lacking.

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The aim of this paper is to define the distribution and polarization of TAMs in GBM, and to review the new current knowledge about this topic. The global scenario of macrophage plasticity in GBM is discussed, suggesting pivotal points for therapeutic applications.

## 2. Materials and methods

### 2.1. Patients and specimens

We retrospectively evaluated specimens resected from 37 patients who underwent tumor resection during the period 2001–2014. The cohort of patients consisted of 22 males and 15 females, with a mean age of 62 years (range 41–78 years). Informed written consent was obtained from all patients. All tumors were histologically assessed and graded as GBM (WHO grade IV) on formalin-fixed and paraffin-embedded tissue sections by three experienced pathologists (GD, PZ, CM) according to the World Health Organization criteria. Deparaffinized tissue sections (4 μm thick) were used for all the staining procedures employed in our study: hematoxylin and eosin, immunohistochemistry, and immunofluorescence. All procedures were carried out at room temperature.

### 2.2. Immunohistochemistry

All cases were tested for CD68 (mouse, 1:1500; Dako), inducible nitric oxide synthase (iNOS) (mouse, 1:100; Sigma), CD163 (mouse, 1:200; Leica Microsystems) and CD31 (mouse, 1:50; Dako) by means of an Autostainer Link 48 Dako. CD68+ cells were considered as M0, M1, and M2 macrophages, CD163+ cells were recognized as M2 macrophages while iNOS+ cells were considered typical M1 macrophages [7–11], when a consistent morphology was also displayed.

The extent of infiltrating CD68+ cells, iNOS+ cells, and CD163+ cells was evaluated in three different areas *per* neoplasia: parenchyma, perivascular area (around bouquets of proliferating microvascular structures), and perinecrotic area. A direct cell count was conducted by using the cell count function in Image J 1.42 [12] software, on five fields per area (parenchyma, perivascular and perinecrotic), for all the cases involved in this study. Each field consisted of a photo obtained at 400X magnification.

### 2.3. Immunofluorescence and confocal analysis

For confocal microscopy, sections were deparaffinized before endogenous peroxidase quenching and heat-induced epitope retrieval. The distribution pattern of M1/M2 macrophages was evaluated by the double staining method for differentiating between CD163+/CD68+ cells and iNOS+/CD68+ cells. After permeabilization and blocking with 100 μL of 0.5% Saponin and 10% BSA in 1× PBS for 30 min, sections were incubated (1–2 h) with primary reagents in the same buffer used for permeabilization. Slides were extensively washed with 1× PBS, then incubated with secondary reagents (FITC-conjugated anti-mouse; 1:400; Santa Cruz Biotechnology), and washed again. Antibodies directed to CD163 and iNOS were directly added to slices, whereas for immunostaining of CD68 (rabbit, 1:100; Aabcam), we used an amplification biotin/streptavidin-based method. In this case, after permeabilization, the slides were sequentially incubated with streptavidin and biotin, using reagents of the avidin/biotin blocking kit (Vector Laboratories), and then processed for immunofluorescence with secondary biotinylated antibody (anti-rabbit; 1:1000; Sigma) and Alexa-Fluor 555-conjugated streptavidin (1:400; Santa Cruz Biotechnology). Sections were counterstained with DAPI (2 μg/mL; Santa Cruz Biotechnology), mounted using an antifade mounting medium (Life Technologies, Monza, Italy), and observed with a Laser Confocal Scanning Microscopy (SP2 LSCM, Leica Microsystems). Single staining for CD68, CD163, and iNOS, as well as negative controls were carried out.

### 2.4. Statistical analysis

A statistical descriptive analysis was performed in order to compare the size of the major macrophage populations reported in our study, CD68+ macrophages and CD163+ macrophages, in three different areas of GBM: tumor parenchyma, perivascular (bouquets of proliferating vessels), and perinecrotic areas. Results are expressed as mean ± SD. In order to verify if there was a significant difference between the number of CD68+ cells and CD163+ cells in the three different areas, a student's *t*-test was separately performed for perinecrotic, perivascular, and parenchymal areas. Moreover, to assess whether a specific distribution of CD163+ and CD68+ cells characterizes the three different histopathological areas, an analysis of variance between groups (ANOVA) for each of these two phenotypes of TAMs was performed. Each test performed was considered significant when $p$ value was $\leq 0.05$.

## 3. Results

### 3.1. Histopathological and immunohistochemical results

Our GBM series comprises grade IV astrocytic tumors, showing atypia, mitotic activity, microvascular proliferation, and (all but five) necrosis. Immunohistochemical evaluation of macrophages phenotype showed an interesting scenario; the number of CD163+ macrophages was significantly higher ($p < 0.001$) than those of CD68+ macrophages in perinecrotic area, parenchyma, and, in particular, in perivascular areas (Figs. 1–3; Table 1). iNOS positivity was not detectable (Fig. 4). We subsequently analyzed the location of macrophage phenotypes and found that the infiltration of CD163+ cells and CD68+ cells, individually evaluated in parenchyma, perivascular, and perinecrotic areas, was significantly different ($p = 0.004$ and $p = 0.033$; Table 2).

### 3.2. Confocal analysis

Double immunofluorescence staining for CD68 and CD163 or iNOS, marking M2 or M1 macrophages, respectively, confirmed the immunohistochemical results. In confocal analysis, the CD163+ population consisted of both CD163+/CD68+ macrophages and single stained CD163+ cells (Fig. 5). Interestingly, such a finding was more evident in perivascular areas (Fig. 5C). Double stained iNOS+/CD68+ macrophages were rare in all GBM analyzed, without a specific distribution among the areas considered in this study (Fig. 6).

## 4. Discussion and review of the literature

TAMs are the most common infiltrating immune cells in malignant glioma and can account for up to 40% of the tumor cell mass [13–15]. TAMs in human gliomas originate from at least two distinct sources: activated resident microglia, derived from embryonic yolk sac myelomonocytes, populating the primitive CNS and peripheral bone marrow-derived mononuclear cells [13,16–18]. Both cell populations contribute significantly to the macrophage content of human gliomas; however, phenotypic and functional differences remain largely unknown [19].

Within the tissues, cells of the monocyte–macrophage lineage display substantial phenotypic diversity and plasticity. Macrophage activation can be divided into a classical (interferon-γ