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THESIS TITLE

# The immunological landscape of primary brain tumors: a comparative study of the immunosuppressive myeloid cell populations in benign and malignant tumors.

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# ABSTRACT

The immune system plays a dual role in cancer progression, either preventing or promoting tumor progression and the fine regulation of the complex interaction between immune system and tumor determines patient outcome. It has been demonstrated that also in brain tumors a combination of signals and soluble factors secreted by tumor, immune and stromal cells are able to potentiate tumor progression. Therefore, the interest towards the characterization of tumor microenvironment in these tumors is growing and the presence of tumor infiltrating immune cells of myeloid origin has already been reported, but a clear phenotypic and functional characterization in human brain tumors still lacking. For this reason, in this research project we performed a deep analysis of both circulating and tumor-infiltrating leukocytes present in meningioma (MNG) and glioblastoma (GBM) patients in order to dissect their properties, with the ultimate goal of finding new immunological therapeutic strategies.

We thus performed an extensive immunophenotyping of peripheral blood and fresh tumor tissue at surgery by multiparametric flow cytometry in 34 patients affected by MNG (WHO grade I-II) and in 76 patients affected by GBM (WHO grade IV glioma), along with immunosuppressive activity of sorted cells of myeloid origin.
In the peripheral blood, we observed a number of significant alterations in myeloid cell subsets indicating a specific monocyte subsets as the main cell subset actively recruited to the tumor. Moreover, four subsets of myeloid-derived suppressor cells (MDSCs) are detectable in the blood and in the tumor tissue of patients affected by MNG and GBM. Three of these subsets are significantly expanded in the blood of patients, whereas two of them are significantly expanded in the tumor tissue.

In addition, we assayed ARG-1 (arginase 1) levels and activity in plasma samples from patients affected by MNG and GBM, and observed both a significantly increased level and a boost of its functional activity, compared to the control group.

At the tumor site, we observed a large leukocyte infiltrate, predominantly constituted by CD33+ myeloid cells, largely composed of macrophages endowed with suppressive activity and significantly expanded in both types of tumor. Based on the expression of different markers, in GBM patients, we were able to discriminate bone marrow-derived (BMDM) macrophages from resident microglia (MG). These populations showed a different suppressive activity, since BMDMs

displayed a higher immunosuppressive activity compared to MG cells that showed low or no suppressive immune regulatory ability.

Taken together the results of this study shed light on the complex interaction between immune system and the main tumors of the brain.

# RIASSUNTO

Il sistema immunitario svolge un duplice ruolo nella progressione del cancro, è in grado sia di prevenire che di promuovere la progressione tumorale e la regolazione della complessa interazione tra sistema immunitario ed il tumore è in grado di determinare la prognosi del paziente. Inoltre, è stato dimostrato che, anche nei tumori cerebrali, una combinazione di segnali e di fattori solubili secreti dal tumore, dalle cellule immunitarie e dalle cellule stromali, è in grado di potenziare la progressione tumorale. Pertanto, in questi tumori sta crescendo l'interesse verso la caratterizzazione del microambiente tumorale ed è già stata dimostrata la presenza di cellule immunitarie di origine mieloide infiltranti il tumore, ma una chiara caratterizzazione fenotipica e funzionale di queste popolazioni non è ancora stata documentata. Pertanto, in questo progetto di ricerca abbiamo eseguito un'analisi approfondita sia dei leucociti circolanti che dei leucociti infiltranti il tumore nei pazienti affetti da meningioma (MNG) e glioblastoma (GBM), al fine di studiarne le caratteristiche, con l'obiettivo finale di trovare nuove strategie terapeutiche.

Abbiamo pertanto eseguito un’accurata immunofenotipizzazione del sangue periferico e del tessuto tumorale, analizzato subito dopo la resezione chirurgica, mediante citofluorimetria a flusso multi-parametrica in 34 pazienti con MNG (grado I-II OMS) e in 76 pazienti con GBM (glioma di grado IV OMS). Abbiamo inoltre testato l’attività immunosoppressiva delle popolazioni di origine mieloide isolate da biopsia.
Nel sangue periferico abbiamo osservato delle alterazioni significative nelle sottopopolazioni di cellule di origine mieloide, rivelando che un particolare sottogruppo di monociti viene attivamente reclutato al sito tumorale. Inoltre, quattro sottopopolazioni di cellule soppressorie di derivazione mieloide (MDSC) sono rilevabili nel sangue e nel tessuto tumorale dei pazienti affetti da MNG e GBM. Tre di queste popolazioni sono significativamente espanse nel sangue dei pazienti, mentre due di esse sono significativamente espanse nel tessuto tumorale.

In questo studio, abbiamo analizzato anche i livelli plasmatici di arginasi 1 (ARG-1) e la sua attività funzionale in campioni di plasma di pazienti con MNG o GBM ed abbiamo osservato sia un aumento significativo della sua concentrazione plasmatica che della sua attività funzionale, rispetto al gruppo di controllo.
Analizzando il tessuto tumorale, abbiamo osservato la presenza di un importante infiltrato leucocitario, costituito prevalentemente da cellule mieloidi $CD33^{+}$ ed in

particolare da macrofagi dotati di attività soppressiva e la cui percentuale è notevolmente elevata in entrambi i tipi di tumore. Nei pazienti con GBM, sulla base dell'espressione di diversi marcatori, abbiamo potuto discriminare i macrofagi derivati dal midollo osseo (BMDM) dalla microglia (MG). Queste popolazioni macrofagiche hanno dimostrato avere una diversa attività soppressoria, infatti, i BMDM risultano essere più immunosoppressivi rispetto alla MG che invece ha una bassa o irrilevante capacità soppressoria.

I risultati di questo studio sottolineano quindi l'esistenza di una complessa interazione tra sistema immunitario ed i principali tumori cerebrali.

# 1. INTRODUCTION

## 1.1 Immune system and cancer: from the concept of immunosurveillance to the theory of the tumor escape

The immunosurveillance theory was formally enunciated in 1957 by Burnet and Thomas (Burnet 1957). This theory claims that malignant transformation naturally occurs in the body as a result of genetic changes, but tumors do not become clinically detectable because they are eliminated by the immune system which recognizes the new antigenic determinants expressed on their surface, that are able to elicit a protective immune response (Burnet 1970). According to this theory, the higher incidence of cancer in immunodeficient or transplanted patients constituted an early evidence. However, the long-term follow up of these patients revealed that this high risk was partially explained by an impaired natural protection against oncogenic virus (Birkeland, Storm et al. 1995). Moreover, subsequent studies carried out on immunodeficient mice (nude mice) showed no higher incidence of tumor development, as compared to immunocompetent mice, making these data to be criticized by the scientific community (Stutman 1974). However, the development of this experimental model presented considerable limitations, even though they were not appreciable in the beginning. Indeed, nude mice are not completely immunodeficient, as considered in the first measure, as they possess myeloid cells, natural killer cells (NK) and B lymphocytes, which can thus provide a reduced, but still sufficient, anti-tumor immunity. In only a few decades, the improvement of tumor immunology knowledge and the important technological discoveries displayed a renewed interest in the theory of cancer immunosurveillance. In particular, the development of transgenic and knock-out mice lacking NK, T and B lymphocytes was finally able to provide strong and convincing data in favor of cancer immunosurveillance, thus proving that the immune system can exert both host-protecting and tumor-sculpting effects on developing malignancies. As a consequence, the initial theory of cancer immunosurveillance was no more appropriate to describe the interaction between tumor and immune system, so Robert Schreiber and collaborators proposed to use the broader term “Cancer immunoediting” to describe the ambivalent behavior of the immune system towards cancer (Dunn, Bruce et al. 2002) (Dunn, Old et al. 2004) (Swann and Smyth 2007).

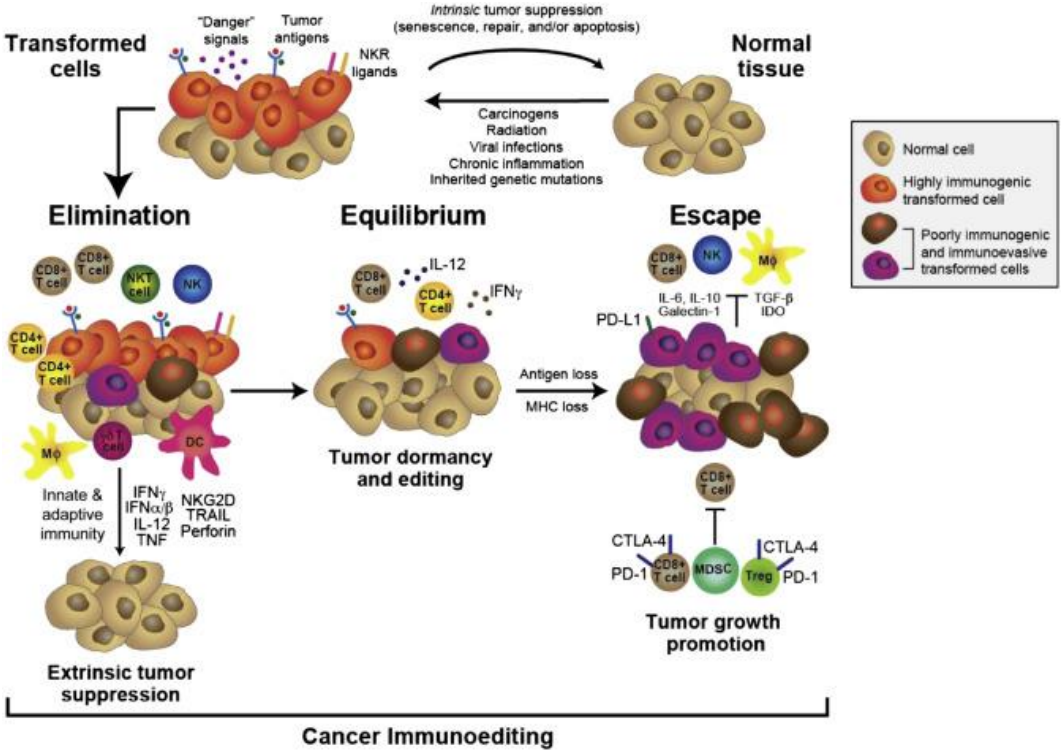



**Fig. 1 The three phases of cancer immunoediting.**

The three Es of cancer immunoediting refers to 'Elimination', 'Equilibrium', and 'Escape'. Elimination: the development of tumor is kept in check by the immunosurveillance. Equilibrium: tumor cells that have survived the immune surveillance are in balance with immunity of the host. Escape: tumor cells grow and overwhelm the control of the host immune system.
(Mitchell, Xie et al. 2008)

Cancer immunoediting can be considered the result of three processes: elimination, equilibrium and escape (Fig. 1), defined as the three "Es" of cancer immunoediting. The first phase, *Elimination*, is the moment in which immunosurveillance takes place and the immune system is able to recognize and eliminate newly formed cancer cells. If this phase is complete, then the arising tumor is eliminated completely by the immune system.

However, if the elimination of the tumor is incomplete, then a second phase takes place, called the *Equilibrium* phase, in which the genetic instability of malignant cells gives rise to low-immunogenic clones that have a higher probability to survive in an immune-competent host and are therefore favored in the Darwinian selection of tumor variants. The alterations that occur during the immunologic sculpting of a developing tumor are probably facilitated by genetic instability of cancer cells. In

the equilibrium phase, the tumor is not yet clinically apparent because the immune system is still able to control the growth of the majority of malignant cells.
If this equilibrium phase breaks, then tumors can escape the immune control. This phase is generally accompanied by the set-up of an immunosuppressive microenvironment that favors tumor growth in an uncontrolled manner. This part is the third phase, and is called "*Escape*" (Dunn, Bruce et al. 2002).

### 1.2 The concept of tumor microenvironment

The tumor microenvironment (TME) contains different populations of leukocytes infiltrating the tumor, having a beneficial as well as a deleterious action on disease progression (Gajewski, Schreiber et al. 2013). Moreover, the tumor microenvironment also contains stromal cells, like fibroblasts, that shape and sustain the complex architecture of cancer, and vascular endothelial cells of blood vessels, supporting the nutritional requirements of the growing mass (Fig. 2).

Most of the cell populations in the TME is composed of infiltrating leukocytes belonging both to innate and acquired immunity (Gajewski, Schreiber et al. 2013). The presence of T cells is quite relevant in the TME, as these cells are characterized by high specificity of action due to their antigen specificity. In particular, CD8+ T cells (cytotoxic T lymphocyte, CTL) are able to destroy target cells upon recognition of antigens presented on major histocompatibility complex (MHC) I molecules by cancer cells. Also, MHC II molecules are able to present tumor antigens to T lymphocytes, recognized by $CD4^+$ T helper (Th) cells, activating a variety of immune cells through ligand-dependent interactions and secretion of cytokines. Immune infiltrates characterized by high $CD8^+$ cells and memory T cells are prognostic of better diseases control, while association between $CD4^+$ cells and clinical outcome is more controversial (Fridman, Pages et al. 2012). Several mechanisms are known to limit T cell functions in the tumor milieu; one of the principal mechanisms of negative T cells regulation is the induction of exhaustion (Ahmadzadeh, Johnson et al. 2009), a process characterized by expression of multiple inhibitory receptors on T cells which impair their activation and effector functions (Wherry 2011), and generation of regulatory

T cells (Treg) capable to directly suppress the T cell response (Lanca and Silva-Santos 2012).

Through short-lived responses, mainly mediated by NK and natural killer T cells (NKT), the innate immunity could contribute to tumor elimination. However, tumors are able to impair also the action of innate cytotoxicity: indeed intra-tumoral NK cells are often anergic (Schleypen, Von Geldern et al. 2003) (Vivier, Ugolini et al. 2012).

Myeloid cells are the leukocytes characterized by the strongest ambivalence. In fact on one side, myeloid dendritic cells and macrophages are potentially capable of efficient antigen presentation and contribute to the immune attack towards malignant cells, but on the other side, in different conditions the same myeloid cells can prevent or even suppress anti-tumor immunity (Gabrilovich, Ostrand-Rosenberg et al. 2012). Beside macrophages and plasmacitoid dendritic cells, myeloid-derived suppressor cells (MDSC) have a key role in the suppression of the anti-tumor immune response and, albeit each subset is provided with specific suppressive machinery, they share some characteristics like the expression of ligands for immune checkpoint molecules (programmed death-ligand 1, PDL-1), the production of suppressive cytokines (interleuchine 10 ,IL-10) and the activation of indoleamine-2,3-dioxygenase (IDO) and arginase-1 (ARG-1), that will be discussed later (Gajewski, Schreiber et al. 2013).

The importance of myeloid cells is demonstrated by their wide array of functions in both the innate and adaptive immune systems and mounting an appropriate response to a given antigenic source. This includes antigen presentation, wound repair, organ development, cytotoxic clearance of pathogens, and tissue remodeling and homeostasis. Furthermore, they influence both the innate and adaptive immune response upon activation through secretion of pro-inflammatory cytokines, pro-fibrotic and angiogenic factors that may ultimately alter tumor development (Curran and Bertics 2012).

An important contribution to the immunological contexture is given by stromal cells like fibroblast, vascular endothelial cells and extracellular matrix (ECM). These components shape the architecture of cancer and can impede immune infiltration

through a direct physical barrier or may contribute to immune evasion actively, participating to immune suppression (Salmon, Franciszkiewicz et al. 2012) (Kraman, Bambrough et al. 2010).

The coordination of this dynamic interplay between immune players, stromal cells and cancer is orchestrated by a variety of cytokines and chemokines. High expression of chemokine (C-X3-C motif) ligand 1 (CX3CL1), chemokine (C-X-C motif) ligand 9 (CXCL9) and C-X-C motif chemokine ligand 10 (CXCL10) is associated with infiltration of effector and memory T cells (Mlecnik, Tosolini et al. 2010) while the C-C chemokine receptor type 4 (CCR4) – C-C motif chemokine ligand 22 (CCL22) axis drives the trafficking of suppressor cells (Obermajer, Wong et al. 2012). Moreover, a recent study indicates that production of reactive nitrogen species within the tumor microenvironment results in nitration of C-C motif chemokine ligand 2 (CCL2) which results in a hindered T cell infiltration (Molon, Ugel et al. 2011). On the other hand, cytokines are more involved in shaping the functional properties of the immune contexture. There are cytokines with direct suppressive function like IL-10 and transforming growth factor beta (TGF-β), while cytokines classically involved in inflammation, like granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-6, could mediate immune suppression through induction of myeloid-derived suppressor cells (MDSCs) (Gabrilovich, Ostrand-Rosenberg et al. 2012) (Marigo, Bosio et al. 2010).

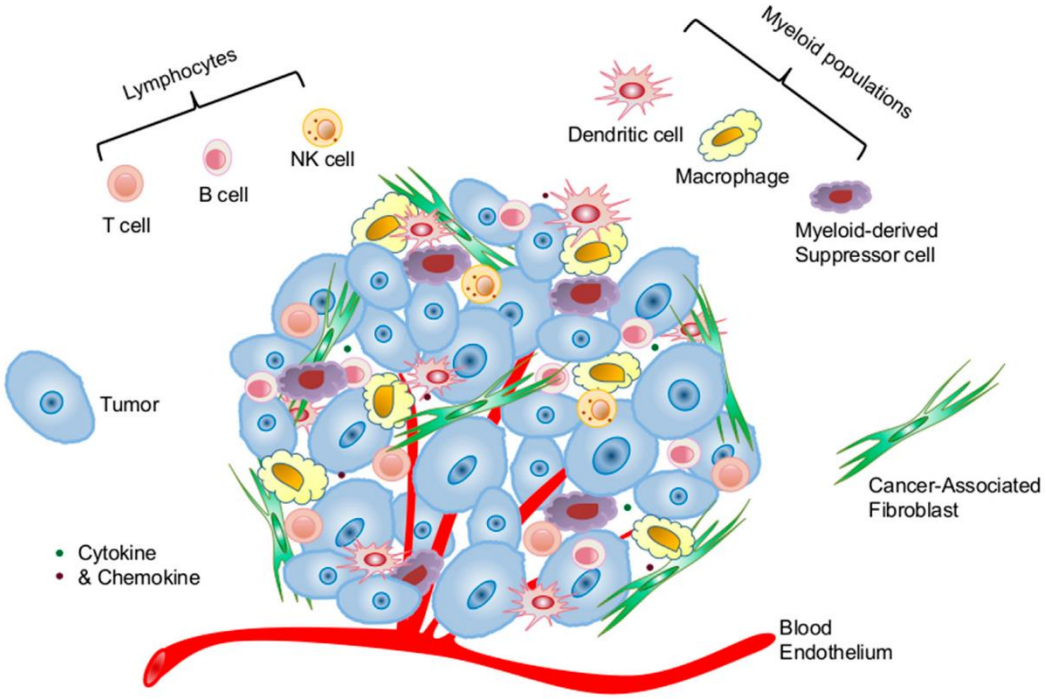



**Fig.2 Cellular constituents of the tumor microenvironment.**

The cellular constituents contain tumor and non-tumor cells. The immune cell compartment includes tumor-infiltrating lymphocytes and tumor-associated myeloid populations. The stromal compartment consists of cancer-associated fibroblasts and endothelial cells of the lymphatic and blood vasculature (Cui and Guo 2016).

### 1.3 Tumor immune suppression mechanisms

In the last phase of cancer immunoediting, tumor cells are able to outgrow because they evade the control of the immune system. One of the mechanisms implemented by the tumor to evade the immune system is represented by an impaired antigen presentation that can be due to: downregulation of the expression of tumor antigens (Maeurer, Gollin et al. 1996) (Vasmel, Sijts et al. 1989), or to reduced MHC-I expression that prevents recognition of tumor cells by the immune system (Hicklin, Marincola et al. 1999) (Garrido, Ruiz-Cabello et al. 1997). The tumor evasion has been proposed as a new hallmark of cancer, representing a significant obstacle to both anti-tumor immunity and cancer immunotherapy (Hanahan and Weinberg 2011). The production of immunosuppressive factors, that can be secreted by the malignant cells themselves or by other cells of the TME

(such as immune, epithelial or stromal cells) is another strategy implemented by tumors (Elgert, Alleva et al. 1998) (Chouaib, Asselin-Paturel et al. 1997).

For example, IL-10 and TGF-β can contribute together with presentation of the tumor antigens by B cells to $CD4^+$ T lymphocytes, to the deviation from a Th1 response, that is required for efficient tumor rejection, toward a Th2 humoral response (Maeda and Shiraishi 1996) (Qin, Richter et al. 1998). Likewise, the depletion of essential nutrients (required by lymphocytes), like L-arginine or L-tryptophan, through activation of ARG-1, inducible Nitric Oxide Synthase (iNOS) or IDO, has immunosuppressive properties (Bronte and Zanovello 2005) (Soliman, Mediavilla-Varela et al. 2010). In fact, the downregulation of the ζ chain in the T cell receptor (TCR) complex and proliferative arrest of antigen-activated T cells is caused by the depletion of these amino acids.

In order to prevent their immune detection and eradication, cancerous cells utilize a variety of immunosuppressive factors including ARG-1. ARG is a manganese-containing enzyme, able to catalyze the hydrolysis of arginine to ornithine and urea. ARG exists in two isoforms, ARG-1 and ARG-2 that share 58% of the amino acid sequence. The two enzymes have different distributions within the cell and are present in different type of cells. ARG-1 (hepatic isoform) has cytoplasmic localization and is present in hepatocytes and in immune cells, while ARG-2 (renal isoform) has mitochondrial localization and is present in tubular cells, cancer cells and T lymphocytes. Furthermore, it has been extensively reported that depletion of arginine by arginase-expressing myeloid cells contributes to an immunosuppressive environment which inhibits proliferation and T cell effector functions (Bronte and Zanovello 2005) (Geiger, Rieckmann et al. 2016).
This amino acid serves as a building block for protein synthesis and as a precursor for multiple metabolites that have strong immunomodulatory properties (Grohmann and Bronte 2010). Recent studies show the collaboration between IDO-1 and ARG-1 (Mondanelli, Ugel et al. 2017). In this study TGF-β increases the expression of both ARG-1 and IDO-1 with slight differences; it promotes the transcription and activity of ARG-1 in a more intense and rapid way. The silencing of the ARG-1 gene or the functional inhibition of the ARG-1 protein (with Nv-hydroxy-nor-Arginine-norNOHA), makes the TGF-β unable to stimulate the transcription of IDO-1, in fact, the ARG-1 derivatives are necessary for TGF-β-dependent stimulation

of the activity of IDO-1. In the absence of TGF-β, L-ornithine is able to stimulate the paracrine and autocrine production of TGF-β and in this way also of IDO-1 and its immunoregulatory activity.

Another strategy used by tumor to evade immune is represented by the change of expression of molecules that mediate apoptosis signaling, like receptor binding cancer antigen expressed on SiSo cells (RCAS1) or Fas ligand (CD95L), to avoid the killing mechanisms by the immune system. Moreover, in order to get rid of the anti-tumor lymphocytes, tumors can adopt killing mechanisms proper of cytotoxic immune cells: this phenomenon is called “tumor counter-attack” (Igney and Krammer 2002). T cells can be neutralized by tumor cells because tumor expresses molecules that implement an inhibitory effect on T lymphocytes, such as B7-H1 (B7 homolog 1), or through the induction of T cell anergy, a process that is induced when a T lymphocyte binds via its TCR to a peptide-MHC complex on the target cell without sufficient co-stimulation. Moreover, many tumors do not express costimulatory molecules and this may lead to anergy of anti-tumor T cells because of a lack of a proper co-stimulation signal.

Through all these mechanisms tumors can modify the TME, thus creating an immunosuppressive milieu in which many cell types are present and whose phenotype and function can be changed in order to favor tumor growth. Among these cells there are tumor-associated macrophages (TAMs) whose high plasticity allows them to switch between the tumoricidal and tumorigenic phenotype in response to environmental cues (Chen and Hambardzumyan 2018) (Biswas and Mantovani 2010). Poor prognosis is associated with their accumulation, because through the release of cytokines, growth factors, ECM-degrading enzymes and angiogenic factors, they promote tumor growth, invasion and metastasis (Mangani, Weller et al. 2017) (Nduom, Weller et al. 2015). In addition, TAMs have been implicated in brain tumor angiogenesis and resistance to anti-angiogenic therapies and may also contribute to the colonization and dissemination of brain metastases (Sevenich, Bowman et al. 2014).

MDSCs represent a major population of regulatory cells that are expanded in different types of cancer, impairing antitumor innate and adaptive immune responses (Gabrilovich and Nagaraj 2009) (Peranzoni, Zilio et al. 2010). The

phenotype of MDSC is not restricted to a single defined myeloid cell population, but rather to a collection of immature myeloid cells, that are responsive to tumor-derived soluble factors. In humans, these phenotypes can be traced in three main subsets: granulocytic MDSCs, or polymorphonuclear MDSC (PMN-MDSCs), monocytic MDSCs (M-MDSCs), and early-stage MDSCs (e-MDSCs) (Solito, Marigo et al. 2014). MDSCs originate from myeloid progenitors in the bone marrow, and are expanded and activated by stromal and tumor-derived factors; the different composition of soluble factors mobilizing MDSCs, dictates the phenotypic features of MDSC subsets observed in patients. Moreover, MDSC level appears to correlate with tumor burden, and thus directly contribute to tumor progression (Solito, Marigo et al. 2014). Indeed, it has been shown that MDSCs are capable of interfering with T cell proliferation through both cell-surface interactions and the release of short-lived soluble mediators (Pinton, Solito et al. 2016). MDSCs are also able to promote tumor growth by acting on cancer cells or by triggering mechanisms responsible for tumor dissemination (Solito, Pinton et al. 2017).

### 1.4 Primary brain tumors

Primary brain tumors are cancers that have their origin in the brain and are named on the base of the cell types from which they originate. They can be benign or malignant: a benign tumor is generally composed of cells that develop slowly, without invasive capacity to other parts of the body; in contrast, malignant tumors are composed of cancerous cells that, without proper treatment, grow rapidly and can invade surrounding tissues. Secondary or metastatic brain tumors derive from tumor cells able to spread to the brain from another part in the body. Most often cancers that metastasize to the brain originate from the lung, breast, kidney, colon or from melanomas (Buckner, Brown et al. 2007).

The latest classification of primary brain tumors has been defined in 2016 (Louis, Perry et al. 2016). The commonest central nervous system (CNS) tumors in children are pilocytic astrocytoma, embryonal tumors and malignant gliomas, whereas meningiomas, pituitary tumors and malignant gliomas are most common adult brain tumor types. The degree of tumor malignancy is determined both by the tumor's histopathologic and molecular features. Because of the notable variety and

unusual biology of brain tumors, it has been extremely difficult to develop a widely accepted histological classification system. For this reason, the use of integrated phenotypic and genotypic parameters for CNS tumor classification adds a level of objectivity that has been missing from some aspects of the diagnostic process in the past. The addition of molecular markers provides a greater diagnostic accuracy as that will improve patient management, precision of prognosis and treatment response (Louis, Perry et al. 2016).

### *1.4.1 Gliomas*

Gliomas are the most common type of primary intracranial tumors in adults, representing 31% of all brain and CNS tumors and 81% of malignant brain and CNS tumors (Ostrom, Gittleman et al. 2017). Usually brain tumors are located in the cerebral hemispheres, but could develop in the brain stem, optic nerves, spinal cord, and cerebellum. Gliomas are tumors that derive from glial cells, or precursor cells, and include tumors of astrocytic, oligodendrial, ependymal, or mixed origin. Based on histological and molecular features, the World Health Organization (WHO) classified the different types of gliomas into prognostic grades ranging from I to IV. The most commonly occurring types of gliomas include: astrocytoma (WHO grade I-IV), oligodendroglioma (WHO grade II-III) and oligoastrocytoma (WHO grade II-III) (Louis, Perry et al. 2016). Incidence rates of glioma differ significantly by histologic type, age at diagnosis, gender, ethnicity, and geographic location. In general, gliomas are more common with increasing age, male gender and Caucasian ethnicity (Ostrom, Gittleman et al. 2015). Gliomas make up the largest proportion of malignant brain tumors, but the most common type of malignant primary brain tumor is glioblastoma (glioma grade IV, GBM), which constitutes approximately 47.1% of all malignant gliomas (Ostrom, Gittleman et al. 2017). For the majority of gliomas, no underlying inherited genetic causes have been identified. In fact, linkage studies conducted within families containing multiple affected members have had little success in identifying factors involved in glioma risk, although in a small proportion (about 5-10%) of glioma cases, rare genetic Mendelian cancer syndromes, such as Neurofibromatosis 1 and 2, Lynch syndrome, Li-Fraumeni syndrome and von Hippel-Lindau syndrome, correlated with an increased risk of glioma (Ostrom, Bauchet et al. 2014) (McNeill 2016).

Current WHO brain tumor classification is based on traditional methods that use morphology and histology to subdivide and classify brain tumors and subsequently to assign them a grade based on the presence of mitosis, vascular endothelial proliferation, necrosis and molecular markers. In fact, recent advances in molecular diagnostic techniques provide alternative methods for classifying tumors using molecular abnormalities and signaling pathways involved in carcinogenesis. Subsequent The Cancer Genome Atlas (TCGA) studies allowed to identify further genetic and epigenetic differences able to divide glioblastoma (GBM) into four subgroups with specific aggressiveness, localization and prognosis. Currently, GBM is divided into proneural, neural, mesenchymal and classical forms based on mutations of isocitrate dehydrogenase 1 (IDH1), platelet-derived growth factor receptor A (PDGFRA), neurofibromatosis type 1 (NF1) and epidermal growth factor receptor (EGFR) (Verhaak, Hoadley et al. 2010). In particular, the neural subtype it's the 14.3% of all GBM, while mesenchymal subtype represents the 25% of all GBM characterized by NF1, tumor protein p53 (TP53), cyclin-dependent kinase Inhibitor 2A (CDKN2A) mutation. The proneural subtype corresponds to the 16.9% of all GBM identified by PDGFRA and IDH1 mutation, while classic subtype represents the 26.7% of all GBM defined by phosphatase and tensin homolog (PTEN) mutation, CDKN2A and EGFR amplification. Moreover, the amplification of EGFR and the mutation of NF1 and/or PTEN indicate a more aggressive tumor, while the classical and mesenchymal type have worse prognosis (Lombardi and Assem 2017).

One of the markers that have been recently introduced in the new classification system is the point mutations in isocitrate dehydrogenase 1/2 (IDH1/IDH2). The mutations of IDH1 and IDH2 are very useful for the differential diagnosis of gliomas. The mutational analysis of IDH1/2 can be used to discriminate between primary and secondary (or recurrent) glioblastomas, which are not distinguishable from the histopathological point of view, but from the clinical viewpoint represent two distinct subtypes of grade IV glioma, which develop in different ways and show different prognosis. IDH1/2 mutations also facilitate the distinction of oligodendrogliomas from other types of infiltrating gliomas. Furthermore, the absence of mutations in pilocytic astrocytomas and ependymomas allows to distinguish these gliomas from diffused astrocytomas. Studies related to biomarkers in gliomas have confirmed, in addition to the diagnostic value of the mutations of IDH1 and IDH2, also the

prognostic one. In fact, patients with IDH1 or IDH2 mutated, showed longer survival than patients with IDH wild type (Louis, Perry et al. 2016).

In recent times, a DNA epigenetic modification, that is the methylation of O6-methylguanine, the promoter of the methyltransferase gene (MGMT), has been introduced in the classification criteria of the gliomas. The impact of MGMT methylation on the survival of patients with glioblastoma is a positive prognosis, whereas, for grade II-III gliomas it is unclear (Ostrom, Bauchet et al. 2014).

Recent studies indicate that gliomas can additionally be classified basing on their telomere maintenance mechanisms. Point mutations in the telomerase reverse transcriptase (TERT) gene promoter, leading to increased telomerase activity, are found both in oligodendrogliomas and primary glioblastomas. Gliomas that do not exhibit TERT promoter mutation usually have mutations in the ATRX binding telomere protein, which activates the alternative telomere elongation pathway (ALT). Nearly 75% of the WHO II-III degree astrocytomas and secondary glioblastomas activate this telomerase elongation mechanism independent of telomerase (Ostrom, Bauchet et al. 2014).

### *1.4.2 Meningiomas*

Meningiomas are the most common brain tumors in adults, accounting for 36.8% of all primary brain tumors (Ostrom, Gittleman et al. 2017). These tumors originate from arachnoid cap cells, which are cells covering the arachnoid membrane, one of the three protective layers surrounding the brain and spinal cord. These tumors can grow slowly but they can become very large if left undiscovered and can be severely disabling and life-threatening (Fathi and Roelcke 2013). The incidence of meningiomas increased with age, with a dramatic increase after age 65 years. Moreover, this tumor is more common in women (3:1) and is significantly higher in the Negroid population than in the Caucasian population (Ostrom, Gittleman et al. 2017). Although most meningiomas are benign (grade I, about 81.3% of all meningiomas), the WHO classification system also recognizes atypical (grade II, about 16.9% of all meningiomas) and anaplastic or malignant (grade III, about 1.7% of all meningiomas) meningiomas (Louis, Perry et al. 2016). Most patients develop a single meningioma; however, some patients may develop several tumors that grow simultaneously in other parts of the brain or spinal cord (Kshettry, Ostrom et al. 2015). The most aggressive meningioma types, have a propensity

for recurrence and infiltration of surrounding brain parenchyma. Risk factors for meningioma are not established (Fathi and Roelcke 2013). However, it seems that radiation exposure, as for gliomas, can contribute to the arise of cancer, in addition, neurofibromatosis type 2 (NF2) and Gorlin syndrome appear to be significantly associated with the onset of meningiomas. The most frequent genetic abnormality in meningiomas is genetic loss of chromosome 22.q12.2. This deletion encodes the NF2 gene, whose product, Merlin protein, is involved in the regulation of cell-to-cell contact and motility. The frequency of the NF2 mutation has the same incidence in WHO grade I-III meningiomas, suggesting that NF2 is involved in the initiation of the meningioma rather than in its progression (Fathi and Roelcke 2013). Surgical resection is the standard treatment for symptomatic and growing tumors. Radiation therapy may be an option if the tumor cannot be treated effectively through surgery while chemotherapeutic agents have been reported to be ineffective (Fathi and Roelcke 2013).

### 1.5 The theory of immune privilege

The immune system of the CNS has several distinct characteristics that make it different compared to the peripheral immune system, such as the absence of MHC-II expression by CNS parenchymal cells, except the microglia cells, (Vagaska, New et al. 2016), the blood-brain barrier (BBB) and the anti-inflammatory attribute of the CNS tissue environment, which cause the specialized CNS inflammatory responses that are essential for the conservation of the fragile non-regenerating tissue aspect in the CNS (Louveau, Smirnov et al. 2015).

Certain sites of the human body have immune privilege, meaning they are able to tolerate the introduction of antigens without eliciting an inflammatory immune response. Tissue-grafts are normally recognized as foreign antigen by the body and attacked by the immune system. However, in immune privileged sites, tissue grafts can survive for extended periods of time without rejection occurring. The concept of CNS as an "immune-privileged" organ system, however, has been challenged and re-evaluated over the last twenty years. Generally, in normal (uninjured) tissue, antigens are taken up by antigen presenting cells (dendritic cells), and subsequently transported to the lymph nodes. Alternatively, soluble

antigens can drain into the lymph nodes. In contrast, in the CNS, dendritic cells are not thought to be present in normal parenchymal tissue or perivascular space although they are present in the meninges and choroids plexus. Thus, the CNS is thought to be limited in its capacity to deliver antigens to local lymph nodes and cause T-cell activation (Engelhardt, Vajkoczy et al. 2017). However, the CNS parenchyma has no conventional lymphatic vessel, and although a well-regulated BBB controls the entry of solutes into the CNS, there is still a need to maintain homeostasis and effective afferent pathways to lymph nodes. The cerebrospinal fluid (CSF) drains along lymphatic vessels coming from the cervical district, pass through the cribriform plate and the dura mater, and arrives at the lumbar lymph nodes (Aspelund, Antila et al. 2015). In this way, this pathway allows for trafficking of immune cells.

In the past it was thought that the brain was an immune-privileged site, because of the protective structure of the blood-brain barrier and his behavior. In contrast, now it has been shown that the CNS, including the brain, is more immune competent than previously thought. The immune system is highly active in the brain and interacts with brain tumors. These findings have opened up the possibility to explore the immunotherapy for treating malignant brain tumors.

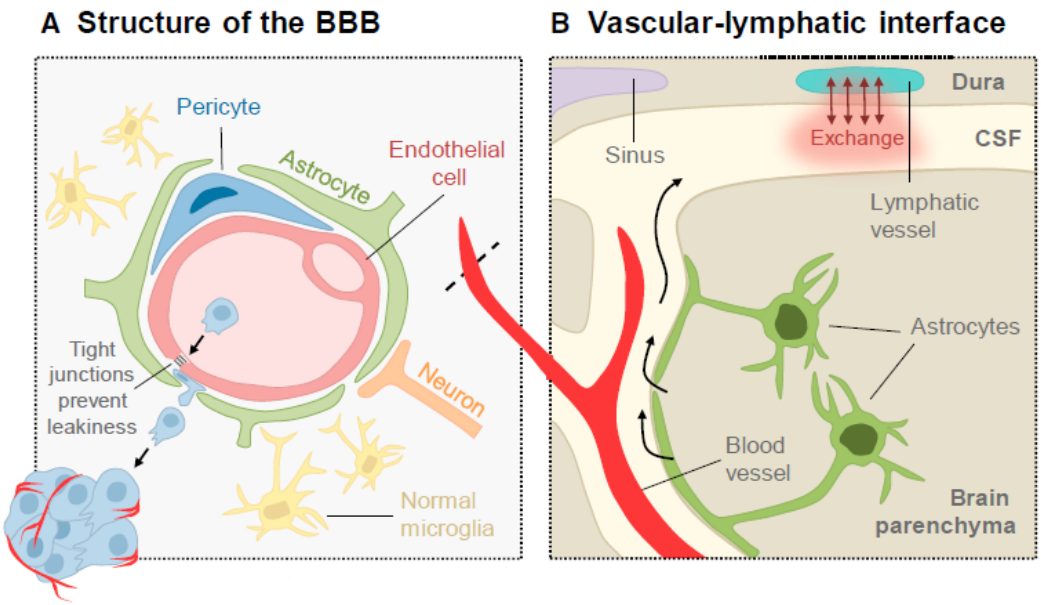


**Fig. 3 Blood and lymphatic vessels of the CNS.**
A) The BBB serves to protect the brain from inflammation and systemic insults. It is composed of endothelial cells, pericytes and astrocytes, which tightly seal the endothelium to regulate permeability. Microglia and neurons can additionally contribute to regulation of BBB integrity. Breakdown of junctional integrity can increase permissiveness to seeding of brain metastatic cancer cells. B) The lymphatic vessel exchange fluid with the CSF that surrounds the brain parenchyma, explaining t how immune cells are trafficked into and out of the brain.
Modified from (Quail and Joyce 2017)

### *1.5.1 The Blood-Brain-Barrier*

The univocal distinction of the brain from other organs is the presence of the blood brain barrier (BBB), that provides a selective barrier between the systemic circulation and the brain. The BBB is meant to protect this essential organ from infection and toxic substances, but this kind of protection simultaneously limits the delivery of many therapeutic agents. However, various brain pathologies, including cancer, can display a loss of BBB integrity resulting in loss (Quail and Joyce 2017).

The BBB is a highly selective semipermeable border that separates the circulating blood from the brain and extracellular fluid in the CNS. The BBB is composed of specialized endothelial cells, pericytes, and astrocytic foot processes, which dictate junctional integrity. An additional contribute to BBB integrity regulation and specifically repairmen of the BBB following injury, can come from microglia. This system allows the passage of water, some gases, and lipid-soluble molecules by passive diffusion, as well as the selective transport of molecules such as glucose and amino acids that are crucial to neural function. This "barrier" results from the selectivity of the tight junctions between endothelial cells in CNS vessels, which restricts the passage of solutes. At the interface between blood and the brain, endothelial cells are stitched together by these tight junctions, which are composed of smaller subunits, frequently biochemical dimers, that are transmembrane proteins such as occluding, claudins and junctional adhesion molecule (JAM) (Daneman and Prat 2015).

While the BBB restricts the entry of many molecules and cells, it is not an impenetrable barrier to transmigration of metastasizing cancer cells. Indeed, in this regard, it has been proposed that the brain can function as a sanctuary site for metastatic cells that effectively breach the BBB, where they are subsequently

protected from the effects of chemotherapy and other agents that cannot penetrate the brain (Eichler, Chung et al. 2011).

### *1.5.2 Lymphatic Vessels*

The lymphatic system connects the circulatory and immune systems; this connection works in sync with blood vessels to operate the exchange of fluid, waste, debris, and immune cells within tissues. Until recent times, it was a common belief the fact that the brain lacked of a classical lymphatic drainage system, partially explaining its immune privilege. However, it has been assumed that the brain immune-surveillance, under normal homeostatic conditions, occurs mainly in the meningeal compartment. Furthermore, detecting lymphocyte and tumor cell passing from the brain to the cervical lymph nodes suggests that there could be a direct route of passage into the lymphatic system (Louveau, Smirnov et al. 2015).

Initial efforts were focused on the identification of the mechanisms of CNS immune surveillance and T cell trafficking in and out of the meningeal region, which envelops the brain and spinal cord. Functional lymphatic vessels were unexpectedly found along the dural sinuses and meningeal arteries during these analyses, making clear that they serve as conduits to the cervical lymph nodes to exchange fluid and immune cells with the cerebral spinal fluid (Aspelund, Antila et al. 2015). In the CNS interstitial fluid culverts through perivascular channels into the CSF. This connection allows macrophages and other antigen-presenting cells (APCs) in the subarachnoid space (SAS) to obtain the antigens in the CNS. Again, antigens released within the CNS are drained towards peripheral lymphoid tissue and can be presented by APCs to naïve T cells, which can subsequently be activated and upregulate the expression of α4 and β1 integrins. When T cell expressing these integrins, they can interact with vascular cell adhesion molecule (VCAM)-1 expressed on the cerebral endothelium and pass across the BBB (Engelhardt, Vajkoczy et al. 2017). The discovery of the dural lymphatic route (also called glymphatic system), suggests that the brain cannot be seen as an immune-privileged organ but rather an accessible site for immunotherapeutic approaches (Louveau, Plog et al. 2017).

### 1.6 Cellular crosstalk in brain tumor microenvironment

The TME appears a critical regulator of cancer progression in primary and metastatic brain malignancies. TME contains an array of neoplastic and non-neoplastic cells, including infiltrating and resident immune cell, vascular cell, and other glial cells, that can influence the tumor development. A reciprocal interaction has been shown between these cells and neoplastic tumor cells to promote tumor growth and progression. The immune cells perform cancer immune surveillance at early stage of premalignant lesion. However, during cancer development, when the immune system is overcome by tumor burden, cancer can escape this surveillance and become incontrollable. Cancer cells can recruit these immune cells changing its functionality, converting the immune system from protective to detrimental to the host. Indeed, in the TME, the immunosuppressive activity of tumor cells potentiates the proliferation and expands the characteristics of brain tumors including migration to borders of normal tissue and invasion, which is associated with degradation of ECM. Moreover, activation of immune cell produces inflammatory factors that can be cytotoxic and cause neurodegeneration (Herz, Filiano et al. 2017).

Another peculiarity on the primary brain tumors is that they are highly vascularized tumors. Overexpression of proangiogenic factors within tumor environment (such as vascular endothelial growth factor, VEGF) leads to the formation of new blood vessels promoting hypoxic environment, necrotic areas, and disruption of BBB integrity, through alteration of endothelial cells and poor recruitment of pericytes. Indeed, dissemination of immunosuppressive agents like secreted factors and cancer related cells in body circulation, is conveyed by blood flow (Quail and Joyce 2017).

#### *1.6.1 Tumor cells*

Glioma cells contribute to the immunosuppressive microenvironment through the secretion of various soluble factors. The first identified immunosuppressive molecules released by glioma cells was the TGF-β2; this molecule has suppressive effects on IL-2 dependent T-cell growth (Schwyzer and Fontana 1985).

In the presence of TGF-β, $CD4^+$ T cells up-regulate FoxP3 and differentiate into Treg cells. Furthermore, TGF-β in effector $CD8^+$ T cells is able to inhibit the expression of several genes, such as interferon-γ (IFN-γ) and granzyme B, which

are necessary for the generation of a powerful antitumor immune response. Moreover, TGF-β can polarize macrophages into a pro-tumorigenic phenotype, and impair antigen presentation by dendritic cells (DC). TGF-β has been regarded an attractive therapeutic target, because of its strong immunosuppressive properties and other further effects contributing to the malignant phenotype of glioma cells such as increased migration and invasion, angiogenesis and proliferation (Mangani, Weller et al. 2017).

Beyond TGF-β, many other glioma cell-secreted factors have been discovered. It has been shown that VEGF it is not only involved in glioma angiogenesis but can also inhibit the maturation and function of DC and induce the expression of inhibiting receptors, such as programmed cell death-1 (PD-1) on activated T cells (Voron, Colussi et al. 2015). As well as in glioma cells, PD-L1 is expressed on immune cell, and it has been shown to be able to inhibit T cell activation and induce T cell apoptosis. Thus, a barrier against cytotoxic T cell can be generated by PD-L1-expressing tumors, allowing maintenance and immunological tolerance (Keir, Butte et al. 2008).

Other factors and cytokines with immunosuppressive functions released by glioma cells are IL-10, regeneration and tolerance factor (RTF), macrophage-colony stimulating factor (M-CSF), nitric oxide (NO), prostaglandin E (PGE), CCL2 and ARG-1. IL-10 may induce the expression of PD-L1 on monocytes while RTF was found to inhibit the ability of NK cells to lyse target cells. Levels of arginase I in the serum have been associated with immunosuppression in GBM patients whereas adult neural stem cells-derived NO can suppress T cell function along with PGE (Mangani, Weller et al. 2017).

CCL2 is a chemokine involved in the migration and tissue infiltration of immune cells, such as monocytes and macrophages. It emerged that the over-regulation of C-C chemokine receptor type 2 (CCR2) in macrophages and Treg cells is determined by CCL2 expression by GBM, with their subsequent accumulation in the tumor environment and inhibition of cytotoxic T lymphocyte activity (Crane, Ahn et al. 2012).

Of note, some glioma-secreted cytokines may have both immune stimulatory and inhibitory roles depending on the context. For example, M-CSF, which functions as a cytokine and growth factor for granulocytes and monocytes, can MDSCs. Moreover, these secreted factors may be upregulated after gliomas undergo conventional therapy (Kohanbash, McKaveney et al. 2013).

Neurons may also contribute to tumor initiation and progression. It is known that mitogenic signals within the brain microenvironment, in order to stimulate growth of neuronal and oligodendroglial precursor cells, can be provided by neurons.
This characteristic suggests that neuronal-specific processes involved in synaptic transmission may have underappreciated mitogenic effects on brain tumors, and that neurotransmitters may inadvertently act as oncometabolites (Quail and Joyce 2017).

### *1.6.2 Tumor Infiltrating Lymphocytes*

Although tumor Infiltrating Lymphocytes (TILs) were found in around 30% of primary brain tumors, several studies failed to establish any correlation with clinical prognosis, especially regarding $CD4^+$ cells and $CD8^+$ cytotoxic T-cells that represent the predominant cell subtypes. The frequency of infiltrating lymphocytes did not vary with tumor grade (Kmiecik, Poli et al. 2013). A study noted a significant correlation between elevated numbers of intratumoral effector T-cells (cytotoxic and helper) and a better survival in primary GBM. Likewise, another study revealed that $CD3^+$ T-cell infiltration was associated with prolonged survival of patients with GBM (Lohr, Ratliff et al. 2011).
It is well established that infiltrating $CD8^+$ cytotoxic T-cells have a key role in anti-tumor activity. More specifically, these T-cells contain several molecules through which they can achieve an anti-tumor function, like perforin, granzymes and tumor necrosis factor (TNF). In patients, the interaction with the brain TME caused re-education of $CD8^+$ T cells. It has been demonstrated that during anti-tumor immunity, the T cell effector function could be altered by local microenvironment (Dietrich, Dutoit et al. 2010).
To prevent their immune detection and eradication, a variety of immunosuppressive mechanisms are used by brain tumors. Patients with primary brain tumors present a dysregulation in the $CD4^+$ T cell fraction, characterized by an expansion of Treg fraction and a reduction in other populations of circulating $CD4^+$ T cells (Wainwright, Sengupta et al. 2011).
It has been demonstrated that Tregs play an integral part in the TME with a fundamental immunosuppressive role. Glioma patients exhibit a decrease in absolute numbers of circulating Th cells, while, there is an increased proportion of immunosuppressive Tregs. The expansion of the Treg fraction in the peripheral

blood and tumor tissue of glioma patients correlates with tumor grade and poor prognosis (Fecci, Mitchell et al. 2006) (El Andaloussi and Lesniak 2007).
The glioma microenvironment efficiently recruits Tregs from the periphery, mainly through the expression and release of CCL2 from the glioma cells, making this molecule a possible therapeutic target. Furthermore, the removal of the Treg fraction from T cells obtained from GBM patients has been shown to restore T cell proliferation to normal levels. Moreover, the in vitro depletion of Tregs from peripheral blood determine both the restoration of the proliferating capacity of the effector T cells and the switch of the cytokine profile produced by Th2 to Th1 (Mangani, Weller et al. 2017).
Therefore, these data seem to indicate that Tregs are not only able to prevent antitumor immune activation in the initial phases, but may also mediate in subsequent states of neoplastic development by inhibiting effector T cells in the tumor microenvironment; this makes them a promising therapeutic target (Quail and Joyce 2017).

### *1.6.3 Myeloid subsets in the CNS*

The CNS and its meningeal coverings accommodate a diverse myeloid compartment contains several sets of myeloid cells that participate in the response to tissue damage and pathogens, in addition to performing specialized functions pertinent to specific tissues.

DCs, acting as messengers between the innate and the adaptive immune systems, are potent antigen presenting cells (APCs) that stimulate T cell responses. Their main function is to process antigen material and to present it on the cell surface to the T cells of the immune system (Banchereau and Steinman 1998).
DCs are present in tissues that are in contact with the external environment, such as the skin, lungs, stomach and intestines. Once activated, they migrate to the lymph nodes where they interact with T cells and B cells, initiating and shaping the adaptive immune response (Banchereau and Steinman 1998).
In the brain, early studies demonstrated that microglia (MG) are the predominant APCs, and that DCs play a less-prevalent role (Quail and Joyce 2017). DCs can be found in the human choroid plexus and meninges. The accumulation of numerous APCs around lymphatic vessels in the meninges strongly suggests that these vessels might serve as important pathways for DC migration in inflammatory

diseases, but their function under homeostatic conditions, however, is not understood (Louveau, Smirnov et al. 2015).

Macrophages are specialized cells involved in the detection, phagocytosis and destruction of bacteria and other harmful organisms. In addition, through the release of cytokines, that activate other cells, they can initiate inflammation and present antigens to T cells. The substantial heterogeneity among each macrophage populations is due to the degree of specialization required in the various tissue; this heterogeneity also moves changes in the morphology, the type of pathogens they can recognize, as well as the levels of inflammatory cytokines produced.

The majority of immune cells within brain tumors are macrophages, often comprising up to 30% of the tumor mass. Distinct macrophage populations exist within the brain TME, including both tissue-resident MG and bone marrow-derived macrophages (BMDMs).

MG represent the brain's most prominent immune cells, which are located in the parenchyma and are tissue-resident macrophages that participate in the development of neuronal circuits, maintenance of synapses, and neurogenesis. Moreover, MG are able to detect changes in pH, purines, cytokines, chemokines, amino acids, and inorganic compounds, because of the dynamicity of their processes and continuous sampling of the environment. Considering their vital role in normal brain function, it is not surprising that MG dysfunction are linked to some neurological disorders (Prinz, Priller et al. 2011).

In the past few years, a series of fate-mapping studies, lineage-tracing experiments, together with parabiosis experiments and neonatal bone marrow transplantations in mouse models, have definitively shown the ontogeny of MG. The current point of view is that, during development, erythroid-myeloid precursors coming from the yolk sac, travel to the brain and differentiate into MG. These cells are not replenished postnatally through peripheral mononuclear hematopoiesis, because they embryonic origin (Ginhoux, Greter et al. 2010). Therefore, the maintenance of MG in the normal adult brain is thought to occur through prolonged cellular longevity and local proliferation (Askew, Li et al. 2017). Similarly, non-parenchymal macrophages within the CNS, like meningeal macrophages, choroid plexus macrophages and perivascular macrophages, arise during embryonic development, and are largely stable population in adult life (Goldmann, Wieghofer

et al. 2016). By contrast, in response to perturbations of tissue homeostasis or pathological conditions, circulating monocytes are recruited to the brain parenchyma and give rise to BMDMs (Shi and Pamer 2011) which exit from the blood flow extravasating the brain endothelial wall and infiltrate the tumor mass (Weiss, Miller et al. 2009) (Fig. 4).

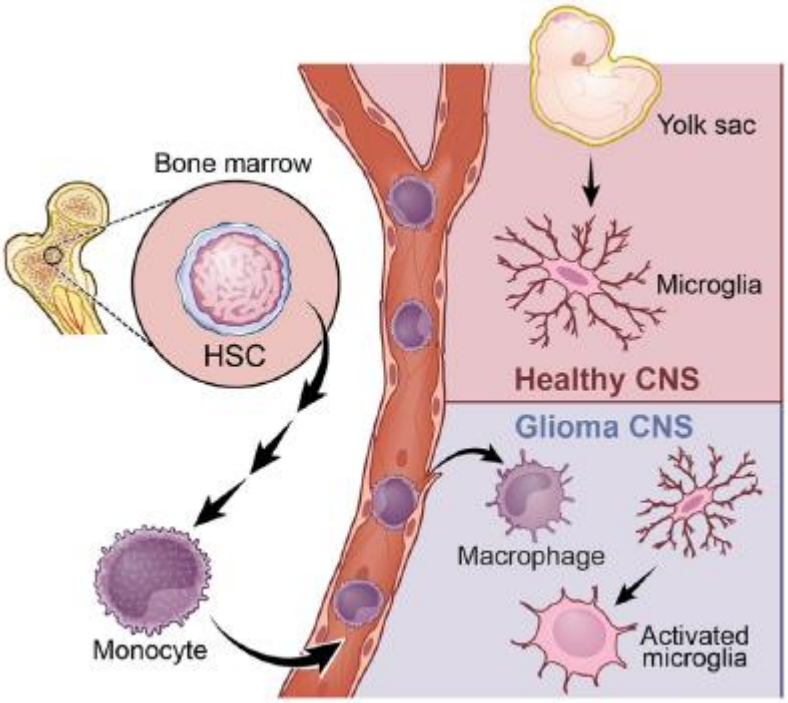



**Fig. 4 The origin of MG and BMDM.**

MG cells originate from yolk sac during embryonal development, while BMDM arise from extravasation of monocyte in the brain after the impairment of the BBB.

Modified from (Chen and Hambardzumyan 2018)

The vast majority (~85%) of TAMs are infiltrating BMDMs, whereas resident MG account for the remaining ~15% (Quail and Joyce 2017) (Chen, Feng et al. 2017). BMDMs within the brain show to be pro-tumorigenic and immunosuppressive and accumulate as the tumor grade increases. Furthermore, analyses of tissue of GBM patients, indicate that these macrophages produce various molecules such as TGF-β, VEGF, epithelial growth factor (EGF) and matrix metalloproteinase, which can promote tumor cell immune evasion, invasion, proliferation and angiogenesis (Mangani, Weller et al. 2017) (Nduom, Weller et al. 2015). Moreover, they are characterized by the secretion of low levels of pro-inflammatory cytokines and by the lack of the expression of key molecules involved in T cell co-stimulation (CD40,

CD80, and CD86), suggesting that they are poor inducers of T cell responses in glioma (Hussain, Yang et al. 2006). Furthermore, this population produce CCL22, which attracts Treg cells that inhibit T cell activation. Additionally, they can also cause T cell apoptosis through their expression of PD-L1, which binds to its receptor PD-1 on activated T cells (Chen and Hambardzumyan 2018).

Classically, MGs and BMDMs have been distinguished by morphological evaluation coupled to immunohistochemical myeloid markers (Roggendorf, Strupp et al. 1996) or by differential expression level of the cell surface marker CD45, where MG being $CD45^{low}$ and BMDMs $CD45^{high}$. In gliomas, the $CD45^{high}$ population represents the largest fraction, even though MGs can upregulate CD45 levels during inflammatory conditions precluding a definite distinction (Mangani, Weller et al. 2017).

In recent years, more accurate cell surface markers for flow cytometry for the discrimination of MG and BMDMs have emerged. For example, both in human brain tissue and mouse models of inflammation, transmembrane protein 119 (Tmem119) is enriched on MG, but not on BMDMs (Bennett, Bennett et al. 2016). Another molecule, CX3C chemokine receptor 1 (CX3CR1), is also expressed by normal MG, but not by macrophages derived from lung, peritoneum, or spleen. However, in brain tumors, subsequent studies have demonstrated that when peripherally-derived macrophages enter in the TME, they up-regulate ‘‘microglia-specific’’ genes including CX3CR1, making this marker inaccurate in the discrimination of the two macrophage populations at the tumor site (Gu, Dean et al. 2009). Recently, in the context of brain malignancy, it was identified Integrin Subunit alpha 4 (CD49D/ITGA4) as a marker for BMDMs but not MG, thus enabling accurate discrimination and isolation of these distinct cell types in cancer patients (Bowman, Klemm et al. 2016) (Zong 2017).

These new microglia-specific markers should allow for the generation of novel and much needed tools for observing and manipulating microglial functions without affecting other cell types.

MDSCs represent another cell population with immunosuppressive properties, expanded both in blood and at tumor site of primary brain tumor patients. Their presence is associated with an overall reduced number of TILs in human gliomas (Raychaudhuri, Rayman et al. 2015), and an increased number of MDSCs correlated with increased glioma grade (Gabrusiewicz, Rodriguez et al. 2016). In

human, M-MDSC have a phenotype characterized by the expression of various markers and we can generally identify them as $CD15^-$ $CD11b^+$ $CD14^+HLA\text{-}DR^{low/-}$ $CD14^+IL4R\alpha^+$ cells. On the contrary, PMN-MDSC are mainly described as $CD14^-$ $CD11b^+$ $CD15^+$(or $CD66b^+$) $CD15^+IL\text{-}4R\alpha^+$. Instead, e-MDSCs are characterized as $Lin^-$ (CD3/14/15/19/56) HLA-$DR^-$ $CD33^+$ (Solito, Pinton et al. 2017).

MDSCs promote tumor growth by acting on cancer cells or by triggering immunosuppressive mechanisms responsible for tumor dissemination, who which can be grouped into four classes (Gabrilovich, Ostrand-Rosenberg et al. 2012). The first type of mechanism is the depletion in the TME of essential nutrients required by lymphocytes, like L-arginine or L-tryptophan, through activation of ARG-1, iNOS or IDO, respectively. The depletion of these amino acids causes downregulation of the ζ-chain in the TCR complex and proliferative arrest of antigen-activated T cells.

The second type of mechanism is the generation of oxidative stress, through a coordinated activation of ARG-1, iNOS and NADPH oxydase that leads to production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Peroxynitrite and hydrogen peroxide hamper the correct function of T cell receptor, block proliferation of T lymphocytes through the loss of TCR ζ chain expression and interfering with IL-2 receptor signaling.

The third type of mechanism interferes with lymphocyte trafficking and viability. Expression of disintegrin and metalloproteinase, like ADAM Metallopeptidase Domain 17 (ADAM17), at the plasma membrane of MDSCs decreases the expression on the surface markers, thereby limiting T cell recirculation to lymph nodes. Furthermore, the production of peroxynitrite induced by MDSC impairs the modification of CCL2, which prevents the migration of effector $CD8^+$ T cells into the tumor nucleus.

The fourth type of mechanism is the activation and expansion of Treg cell populations. MDSCs promote the clonal expansion of antigen-specific natural Treg cells and also induce the conversion of naive $CD4^+$ T cells into induced Treg cells.

In summary, myeloid compartment-derived cells, which are present in primary brain tumor patients, have overlapping functions and intrinsic phenotypic plasticity which could be considered an attractive therapeutic target when designing future trials combining conventional therapies with novel immunotherapeutic approaches.

# 2. AIM OF PROJECT

The tumor microenvironment plays a relevant role in tumor progression: a complex combination of signals and soluble factors secreted by tumor, immune and stromal cells are able to potentiate the proliferation and expand the characteristics of brain tumors, including migration to borders of normal tissue and invasion, which is associated with degradation of extracellular matrix. The presence of tumor infiltrating immune cells has been reported in primary brain tumors, but a clear characterization of the immune landscape is still lacking. For this reason, we analyzed both circulating and tumor infiltrating myeloid cells in order to understand their features and function with the ultimate goal of finding new immunological therapeutic strategies.

To characterize in detail the immune infiltrate composition, we performed an extensive immunophenotyping of peripheral blood and fresh tumor tissue at surgery by multiparametric flow cytometry in patients affected by meningioma (MNG, WHO grade I-II) and by glioblastoma (GBM, WHO grade IV glioma), and sorted the main myeloid cell subsets, to evaluate the functional activity toward activated T cells. In GBM tissues, we also decided to define a strategy to unambiguously identify, sort and characterize tissue resident from blood-derived macrophages.

Moreover, we studied in the plasma of these patients the role of the enzyme arginase-1, to understand its potential role in the immune suppression of these patients.

# 3. MATERIALS AND METHODS

### 3.1 Patients' characteristics

Patients were recruited at the Department of Neurosurgery, Padova University Hospital, Italy. The ethical committee of the IOV-IRCCS and of Padova University Hospital approved all experiments and all patients gave their informed consent. Thirty-four patients with a diagnosis of meningioma were enrolled in the study. We obtained peripheral blood and freshly resected tumor material from 24 patients affected by a WHO grade I meningiomas and 10 patients affected by a WHO grade II meningiomas. One patient had four biopsies, three diagnosed as grade I and one as grade II. Seventy-six patients with a diagnosis of glioblastoma were enrolled in the study. We obtained peripheral blood and freshly resected tumor material from 76 patients affected by WHO grade IV gliomas, including 11 of these were recurrent GBM. As controls, peripheral blood of 35 healthy donors, matched for age and sex, was analyzed.

### 3.2 Blood and tumor samples

From each patient blood sample was withdrawn from the patient the same day of surgery, before anesthesia induction, or the day before surgery, and immediately processed for flow cytometry analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare-Amersham, NJ, USA), followed by dextran sedimentation to obtain PMNs, as previously described (Mandruzzato, Solito et al. 2009).

To produce single-cell suspension, all tumors were processed immediately after resection by enzymatic digestion, using human Tumor Dissociation Kit (Miltenyi Biotec) and following manufacturer's instructions for soft tumors. Before digestion, biopsies were extensively washed with 0.9% sodium chloride solution to remove peripheral blood. The red blood cells remained after enzymatic digestion, were lysed by hypotonic solution of ammonium chloride.

### 3.3 Multiparametric flow cytometry

Peripheral blood was stained with monoclonal antibodies to determine levels of myeloid and T cell subsets. To this aim, 50 µl of fresh blood were washed with PBS plus 1% fetal bovine serum (FBS, Gibco Thermo Fisher Scientific) and subsequently incubated with Fc-Receptor Blocking reagent (Miltenyi Biotec) at 4°C for 10 minutes. Afterwards, cells were stained with anti-IL4R (anti-CD124) PE monoclonal antibody (mAb) and incubated at 4°C for 10 minutes. Then all the other mAbs were added and incubated at 4°C for 20 minutes. At the end of incubation, cells were washed with PBS plus 1% FBS and centrifuged at 1300 rpm for 6 min at 4°C. Red blood cells were lysed using Cal-Lyse whole blood lysing solution (Life Technologies) according to manufacturer's instructions. Blood MDSC subsets were identified by a 7-color staining, containing anti-CD11b Alexa700 (BD Biosciences), anti-CD14 APC-H7 (BD Biosciences), anti-CD15 V450 (BD Biosciences), anti-CD33 PE-Cy7 (eBioscience), anti-α chain of interleukin 4 receptor (IL4Rα) PE (R&D SYSTEMS), Lineage cocktail (anti-CD3, anti-CD14, anti-CD19 and anti-CD56 MiltenyiBiotec) FITC, anti-HLA-DR APC (BD Biosciences) and the immunophenotyping was standardized as reported in (Damuzzo, Solito et al. 2016). Cell suspension obtained from freshly biopsies after enzymatic digestion was labelled with different antibody mixtures optimized to characterize myeloid and lymphocyte cell populations. $5x10^5$ cells were washed with PBS plus 1% FBS and incubated for 10 minutes at 4°C with Fc-Receptor Blocking reagent (Miltenyi Biotec). All the other mAbs were then added and incubated for 20 minutes at 4°C. Subsequently, cells were washed with PBS plus 1% FBS and samples were acquired by flow cytometer. The antibody mixtures used to analyze tumor biopsies contained LIVE/DEAD Fixable Aqua (Life Techonologies), LIVE/DEAD Near IR (Life Technologies), anti-CD45 BV421 (BD Biosciences), anti-CD45 FITC (Miltenyi Biotec), anti-CD33 PE-Cy7 (eBioscience) or anti-CD33 APC (BD Biosciences), anti-HLA-DR APC (BD Biosciences), anti-IL4Rα PE (R&D SYSTEMS), Lin cocktail 1 FITC (BD Biosciences), anti-CD11b Alexa700 (BD Pharmingen), anti-PD-L1 PE (eBioscience), anti-CD14 APC-H7 (BD Biosciences), anti-CD15 FITC (BD Biosciences), anti-CD3 E-Cy7 (Beckman Coulter), anti-CD8 APC-H7 (BD Biosciences), anti-LAG-3 FITC (AdipoGen), anti-PD1 PE (MiltenyiBiotec), anti-CD49D PE (BioLegend). Data were acquired using a LSRII flow cytometer (BD Biosciences) equipped with 4 lasers (405nm, 488nm,

561nm, 640nm) and analysis was performed by FlowJo software (Three Star Inc). Fluorescence minus one (FMO) controls for HLA-DR, IL4Rα, PD-L1, PD-1 and LAG-3 were used as negative controls. All antibodies used for flow cytometry were titrated in a lot-dependent manner.

### 3.4 Arginase type I ELISA

Plasma samples from meningioma (N=32) patients, glioblastoma (N=71) patients and healthy donors (N=22) were obtained upon previous centrifugation over Ficoll-Paque Plus of peripheral blood. The supernatant was collected and further spun at 1,300 rpm, 4 °C for 6 minutes and stored at -80°C. Plasma level of arginase type I (ARG-1) was analyzed using Arginase Liver Type Human ELISA kit (BioVendor Laboratory Medicine Inc.) following the manufacturer's instructions. Hemolized samples were excluded from the analysis. Samples were assayed in duplicates and ARG-1 concentration was extrapolated from the standard curve.

### 3.5 Arginase activity assay

Plasma samples from meningioma (N=32) patients, glioblastoma (N=71) patients and healthy donors (N=22), obtained as before, were tested for arginase activity by measuring the production of L-ornithine or urea at different pH conditions. The ARG-1 functional activity reaches its highest activity at pH 9.5 and its enzymatic activity is routinely tested in this condition; nevertheless, we decided to test the functional activity of ARG-1 even at neutral pH 7.3, in order to simulate its enzymatic activity under physiological conditions. Briefly, in the L-ornithine assay 25µl of $MnCl_2$ 21.6 mM were added to 30 µl of plasma and the mixture was heated at 55°C for 20 minutes to activate arginase. Then, 150 µl of carbonate buffer (100 mM, pH 10) and 50 µl L-arginine (100 mM) were added and the reaction was further incubated at 37°C for 10 minutes. The hydrolysis reaction converting L-arginine to L-ornithine, was identified by a colorimetric assay after the addition of ninhydrin solution and incubation at 100°C for 30 minutes. Absorbance was acquired at 490 nm. Samples were assayed in duplicates and L-ornithine concentration was derived from a standard curve. In the Urea assay cells were lysed with 50 µl of

lysis buffer (0.1% Triton X-100, 100 µg/ml pepstatin, 100 µg/ml aprotinin, and 100 µg/ml antipain). After 30 minutes on a shaker at 37°C, 60 µl of 25 mM Tris-HCl and 2 mM $MnCl_2$ was added. Arginase was activated by heating the solution for 10 min at 56°C. L-Arginine was hydrolyzed by incubating the lysate with 100 µl of 500 mM L-arginine (pH 9.7) at 37°C for 60–120 min. The reaction was stopped with 800 µl of $H_2SO_4$ (96%)/$H_3PO_4$ (85%)/$H_2O$ (1:3:7). The urea concentration was measured at 540 nM after addition of 40 µl of α-isonitrosopropiophenone (dissolved in 100% ethanol), followed by heating at 95°C for 15–40 min. Samples were assayed in duplicates and urea concentration was derived from a standard curve. Both ARG-1 activity assays are based on a hydrolysis reaction, which converts L-Arginine into L-ornithine or into urea. In the assay that converts L-arginine into L-ornithine we have used ninhydrin as a substrate, while in the urea test we used α-isonitrosopropiophenone. Both functional assays are equivalent, but we decided to use the urea assay to test the functional activity of arginase at physiological pH because, compared to the ornithine assay, the background noise is lower, thus resulting in a better resolution.

### 3.6 Isolation of myeloid cell subsets and test of immunosuppressive activity

To isolated $CD33^+$/HLA-$DR^+$ and $CD33^+$/HLA-$DR^-$ cells present in the tumor, the cell suspension obtained after enzymatic digestion of meningioma biopsies was stained with BD Horizon Fixable Viability Stain 520 (BD Biosciences), anti-CD45 BV421 (BD Biosciences), anti-CD33 PE-Cy7 (eBioscience) and HLA-DR APC (BD Biosciences) and filtered through a 100 µM cell strainer. If many debris were present in digested samples before staining, Debris Removal Solution (Miltenyi Biotec) was used to remove them, following manufacturer's instructions. $CD33^+$/HLA-$DR^+$ and $CD33^+$/HLA-$DR^-$ cell subsets were separated by FACS sorting (MofloAstrios, Beckman Coulter). The purity of each fraction was >90%.
To separate $CD49D^+$/HLA-$DR^+$ cells and $CD49D^-$/HLA-$DR^+$ cells present in the tumor, cell suspension obtained after enzymatic digestion of GBM biopsies was stained with Livedead Aqua (Invitrogen), anti-CD45 BV421 (BD Biosciences), CD49D PE (BioLegend) and anti-HLA-DR APC (BD Biosciences) and filtered through a 100 µM cell strainer. If many debris were present in digested samples

before staining, Debris Removal Solution (Miltenyi Biotec) was used to remove them, following manufacturer's instructions. $CD49D^+$/HLA-$DR^+$ or $CD49D^-$/HLA-$DR^+$ cell subsets were then separated by FACS sorting (BD FACS ARIA III). The purity of each fraction was >90%.

Immunosuppressive activity of myeloid cells isolated from tumor of MNG and GBM patients, was tested on the proliferation of PBMCs isolated from the peripheral blood of healthy donors by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare-Amersham, NJ, USA), as previously described (Solito et al., 2011). PBMCs were stained with 0.5 μM CellTrace™ Violet Cell Proliferation Kit (Invitrogen, Molecular Probes, MA, USA), according to manufacturer's instructions. CellTrace-labelled PBMCs were activated with coated 1 μg/ml anti-CD3 and 5 μg/ml soluble anti-CD28 (BioLegend, CA, USA) for four days and co-cultured in flat bottom 96 or 384 well plates at the 1:1 ratio with myeloid cells separated from biopsies of brain tumor patients. Cell cultures were incubated for four days at 37°C and 5% $CO_2$ in arginine free-RPMI (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 150 μM arginine and 10% FBS (SIGMA-Aldrich), 10 U/ml penicillin and streptomycin and HEPES. At the end of cell culture, cells were harvested, stained with anti-CD3 PE-Cy7 (Beckman Coulter) and analyzed by flow cytometry. T cell suppression rate was calculated with two different methods that consider two diverse aspects of immunosuppression. In fact, the immunosuppression can be detected as a reduction in T cell number ("quantitative" immunosuppression), or on the basis of the reduction in number of cell cycles in CellTrace profile ("qualitative" immunosuppression). Qualitative suppression was calculated by analyzing the percentage of proliferating cells from generation 2 onwards; while quantitative suppression was calculated by analyzing the absolute number of proliferating cells by TruCountTM tubes (BD Biosciences), that permits a quantitative measurement of cells. In both cases data were normalized assuming the proliferation of T cells cultured alone as 100%.

### 3.7 Citological preparation and May-Grünwald-Giemsa (MGG) staining

Cytological analysis of cell populations was performed after cytospins' preparation. Cytospin is a technique used to immobilize cells onto glass microscope slides, by centrifugation through specific cytology funnels. $3\times10^4$ cells were washed in a PBS solution and resuspended in up to 200 µl of this solution. Subsequently, the cells were spun at 800 rpm for 5 minutes by Shandon Cytospin 3 centrifuge and stained using the MGG kit for smears (Bio Optica), following manufacturer's instructions. Cell morphology was examined using an Olympus BX-40 microscope (Leica Microsystems) with Leica lenses at 40X magnification. Pictures were taken using a Leica DFC 295 camera (Vashaw Scientific Inc) and acquired with Leica Application Suite Version 4.1.0 (Meyer Instruments).

### 3.8 Statistical analysis

The Mann-Whitney U-test was used to evaluate statistically significant variations between groups of samples. Differences were considered statistically significant with $P<0.05$. All statistical analyses were performed using the Sigmaplot software (Systat Software Inc., CA, USA). Absence of significance was not reported for brevity. Box Plots show the median, the 25th and 75th percentiles (Q1 and Q3 respectively), whiskers that extend to 1.5 inter-quartile range and outliers reported as dots. The mean value is shown in scatter plots with a line while the whiskers present in the histograms display the standard error.

# 4. RESULTS

### 4.1 Characterization of myeloid cell subsets present in the peripheral blood of patients with primary brain tumors

We performed a phenotypical analysis of myeloid cells present in the peripheral blood of GBM and MNG patients by multicolor flow cytometry and we observed, in GBM ptients, a significant increase in the percentage of monocytes as compared to age and gender matched HDs, thus confirming previous results (Gabrusiewicz, Rodriguez et al. 2016). Moreover, in GBM patients this expansion was significant also compared to MNG patients (Fig. 5A). Interestingly, PMN were significanlty expanded in the blood of GBM anf MNG patients, as compared to the control group, as shown in Fig. 5B. Furthermore, GBM patients showed a significant expansion of this fraction also in comparison to MNG patients. These results suggest that in these patients an increased myelopoiesis is present.

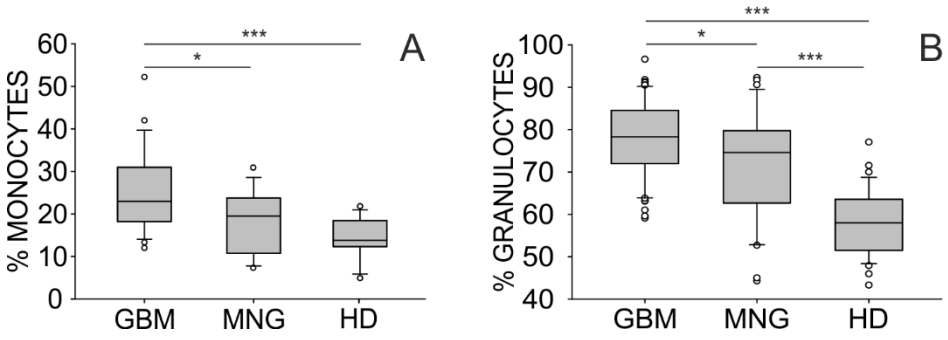



**Fig. 5 Levels of myeloid cells in peripheral blood of primary brain tumor patients.**
Box plot representing the percentage of A) monocytes in blood samples from GBM (N=24), MNG (N=13) patients and HDs (N=12), calculated as HLA-DR$^+$ cells among PBMC, and B) of granulocytes in blood samples from GBM (N=60), MNG (N=31) patients and HDs (N=34), calculated as CD15$^+$ cells among PBL. Mann-Whitney test was performed to compare groups of samples. Asterisks denote significant *P* values: **P*<0.05 and ****P*<0.001.

Given the expansion of monocytes in the peripheral blood of GBM patients, we evaluated in more detail their composition. Based on the expression of the myeloid markers CD16 and CD14 we disitnguished the following monocyte subsets: classical (CD14$^+$/CD16$^-$), intermediate (CD14$^+$/CD16$^+$) and non-classical (CD14$^-$/CD16$^+$). Our results indicate a significant decrease of intermediate monocytes in both MNG and GBM patients as compared to HDs, and also a significant decrease of non-classical monocytes in MNG patients in comparison to patients with GBM (Fig. 6A). Subsequently, we further analyzed the expression of CCR2 on the

surface of these three subsets, since CCR2 is the receptor of CCL2, a chemokine able to attract and accumulate the monocytes at the tumor site. This analysis showed in GBM patients a significant increase of CCR2 expression among the intermediate monocyte subset, as compared to HDs (Fig. 6B). Since classical monocytes have the potential to give rise to intermediate monocytes, and later to non-classical monocytes (Patel, Zhang et al. 2017) and the intermediate monocyte subset express CCR2 on its surface, the decrese of this population in GBM patients suggests its active recruitment to the tumor site.

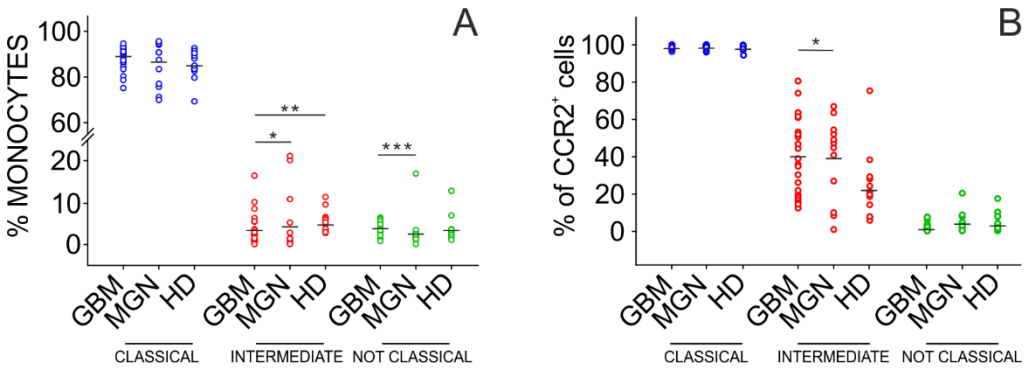



**Fig. 6 Characterization of monocyte subtsets in MNG and GBM patients.**

A) Distribution of classical, intermediate and non-classical monocytes in MNG (n=13) and GBM patients (n=24) in comparison to HDs (n=12). B) Cumulative data showing the expression of CCR2 in monocyte subsets of MNG (n=13) and GBM patients (n=24) compared to HD (n=12). Mann-Whitney U test was performed. Asterisks denote significant P values: *P<0.05, **P<0.01 and ***P<0.001.

Since monocytes are expanded in GBM patients, we decided to evaluate whether monocytic myeloid-derived suppressor cells (MDSCs) are present and we extended the analysis also to granulocytic and to immature subsets of MDSCs. MDSCs are a heterogeneous population of cells that expands during cancer, inflammation and infection, and that has a remarkable ability to suppress T-cell responses. We used a 7-color flow cytometry panel to distinguish in the peripheral blood of 32 MNG and 41 GBM patients the following MDSC subsets:

- MDSC 1: $CD14^{+}/IL4R\alpha^{+}$
- MDSC 2: $CD15^{+}/IL4R\alpha^{+}$
- MDSC 3: $Lin^{-}$/ $HLA\text{-}DR^{-}/CD11b^{+}/CD33^{+}$
- MDSC 4: $CD14^{+}/HLA\text{-}DR^{low/-}$

In this analysis, MDSC 1 and 4 are determined by gating cells on peripheral blood mononuclear cells (PBMC) and they represent a monocytic subset (M-MDSCs). MDSC 2 constitute a granulocytic subpopulation of MDSCs (PMN-MDSCs) and are gated on PMNs, while MDSC 3 represents an immature cell subset, named early-stage MDSC (eMDSCs) evaluated on peripheral blood leukocytes (PBLs) after $CD15^{+}$ exclusion.

As shown in Fig. 7, both MNG and GBM patients had a significant expansion of circulating MDSC 1 (Fig. 7A 9.5±5.7% MNG; 11.4±5.8% GBM), MDSC 2 (Fig. 7B 13.9±5.7% MNG; 18.7±13.9% GBM) and MDSC 4 (fig. 7D 8.2±5.7% MNG; 11.1±6.2% GBM) subsets in comparison to age-matched HDs, while MDSC 3 (fig. 7C) levels were significantly decreased only in GBM patients (0.6±0.6%). Moreover, MDSC1, 2 and 4 levels were significantly higher in GBM patients as compared to MNG.

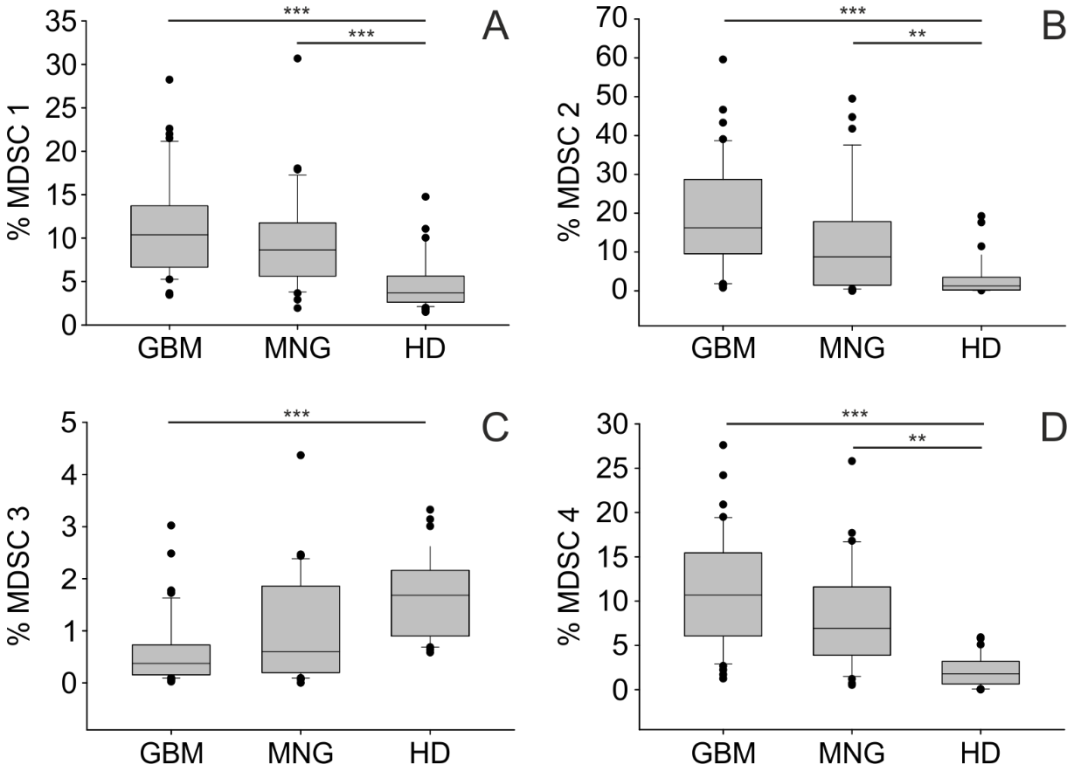



**Fig. 7 Levels of MDSC subsets in the peripheral blood of MNG and GBM patients.**

Box plot reporting the median, the 25th and 75th percentile of MDSC subsets in the peripheral blood of GBM (N=41 for all subsets), MNG (N=32 for MDSC 1 and MDSC 4; N=30 for MDSC 2; N=31 for MDSC 3) patients and HDs (N=35 for all subsets). Mann-Whitney U test was performed. Asterisks denote significant P values: **P<0.01 and ***P<0.001.

### 4.2 Evaluation of the presence and activity of Arginase-1 in the plasma of primary brain tumor patients

It has been extensively reported that depletion of arginine by arginase-expressing myeloid cells contributes to an immunosuppressive environment, which inhibits proliferation, and T cell effector functions (Bronte and Zanovello 2005). Levels of ARG-1 in the serum have been associated with immunosuppression in GBM patients, indicating that MDSCs interfere with T cell proliferation and function (Sippel, White et al. 2011). We thus assayed ARG-1 levels and activity in plasma samples from both MNG and GBM patients and compared these levels to those present in matched HDs. To quantify ARG levels, we used a human ELISA kit, and as shown in Fig. 8A, we observed a significant increase in ARG-1 plasma levels in GBM and MNG patients compared to the HD control group. In order to test the ARG-1 functional activity, we performed a functional assay at alkaline pH and, interestingly, found a significant increase of ARG-1 activity in tumor-bearing patients, compared to HD (Fig. 8B). However, since ARG-1 is released in significant amount in the plasma of these patients, we also tested ARG -1 activity contained in the same plasma samples at pH 7.3. Interestingly, at physiological pH this enzyme displayed a very high and significant activity in GBM patients, and higher than that present in MNG patients, a result that was not appreciated at pH 9.5 (Fig. 8C).

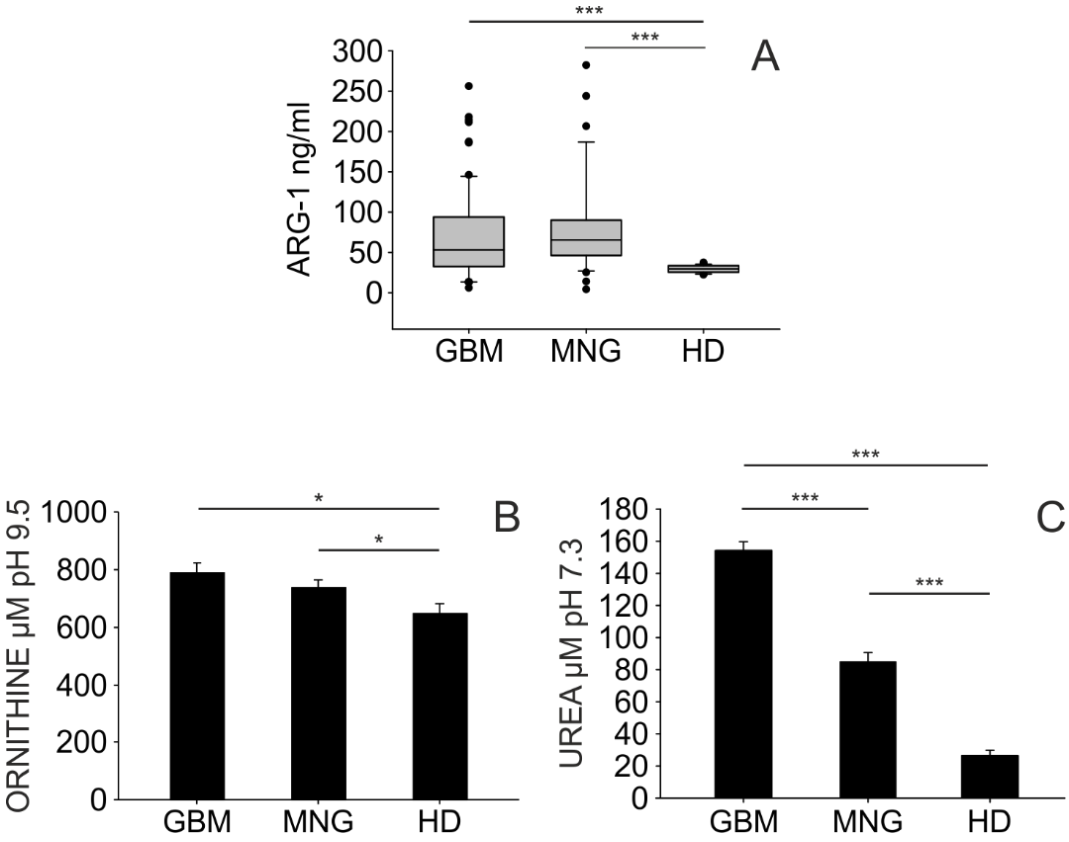



**Fig. 8 Evaluation of ARG-1 levels and activity in the plasma of brain tumor patients.**

A) Box plot indicates the median, the 25$^{th}$ and 75$^{th}$ percentile of ARG-1 levels in the plasma of GBM (N=71), MNG (N=32) patients and HD (N=22). In B) panel, the histograms show the functional activity in the same patients at pH 9.5 while in C) panel the graph represents the ARG-1 functional activity a pH 7.3. Mann-Whitney U test was performed. Asterisks denote significant *P* values: **P*<0.05 and ****P*<0.001.

It thus appears that GBM patients not only have high levels of ARG-1 but also that the enzyme has higher activity at physiological pH. Moreover, although ARG-1 levels and activity at pH 9.5 were comparable with those found in MNG patients, the enzyme present in GBM patients exhibited a significantly higher activity than that of MNG patients, when the test was performed at pH 7.3, thus suggesting that the same levels of protein had a different activity in GBM or in MNG plasma.

### 4.3 Analysis of the composition of leukocyte in MNG biopsies

A central step of this study was characterized by the analysis of myeloid and lymphoid cell infiltrate in meningioma patients, immediately after resection. To this end, we investigated different leukocyte populations infiltrating the tumor tissue, by using multicolor flow cytometry. This evaluation was performed after dissociation of tumor biopsies into single cell suspension, and analysis of the cell composition with 3 cocktails of monoclonal antibodies comprising 14 antigens and identifying 6 cell subsets. In order to characterize the tumor-infiltrating leukocytes, $CD45^+$ cells were gated among live cells, and total myeloid cells were identified as $CD33^+$ among $CD45^+$ leukocytes.

In a cohort of 34 patients, we observed a large presence of live leukocytes, defined as $CD45^+$ (59.5± 25.6%), and, among these, the majority was composed by $CD33^+$ myeloid cells (79.8± 14.6%). In order to discriminate myeloid populations, we used the marker HLA-DR, that allowed us to stratify myeloid cells in two different subsets, $CD33^+$/HLA-$DR^+$ (macrophages) and $CD33^{dim}$/HLA-$DR^-$ (PMNs) cells. The macrophage population represents the 80.4± 20.6% of total $CD33^+$ cells while PMN the 16.2± 17.6%. We confirmed the phenotype of these cell populations with a morphological evaluation through a cytospin (Fig. 9).

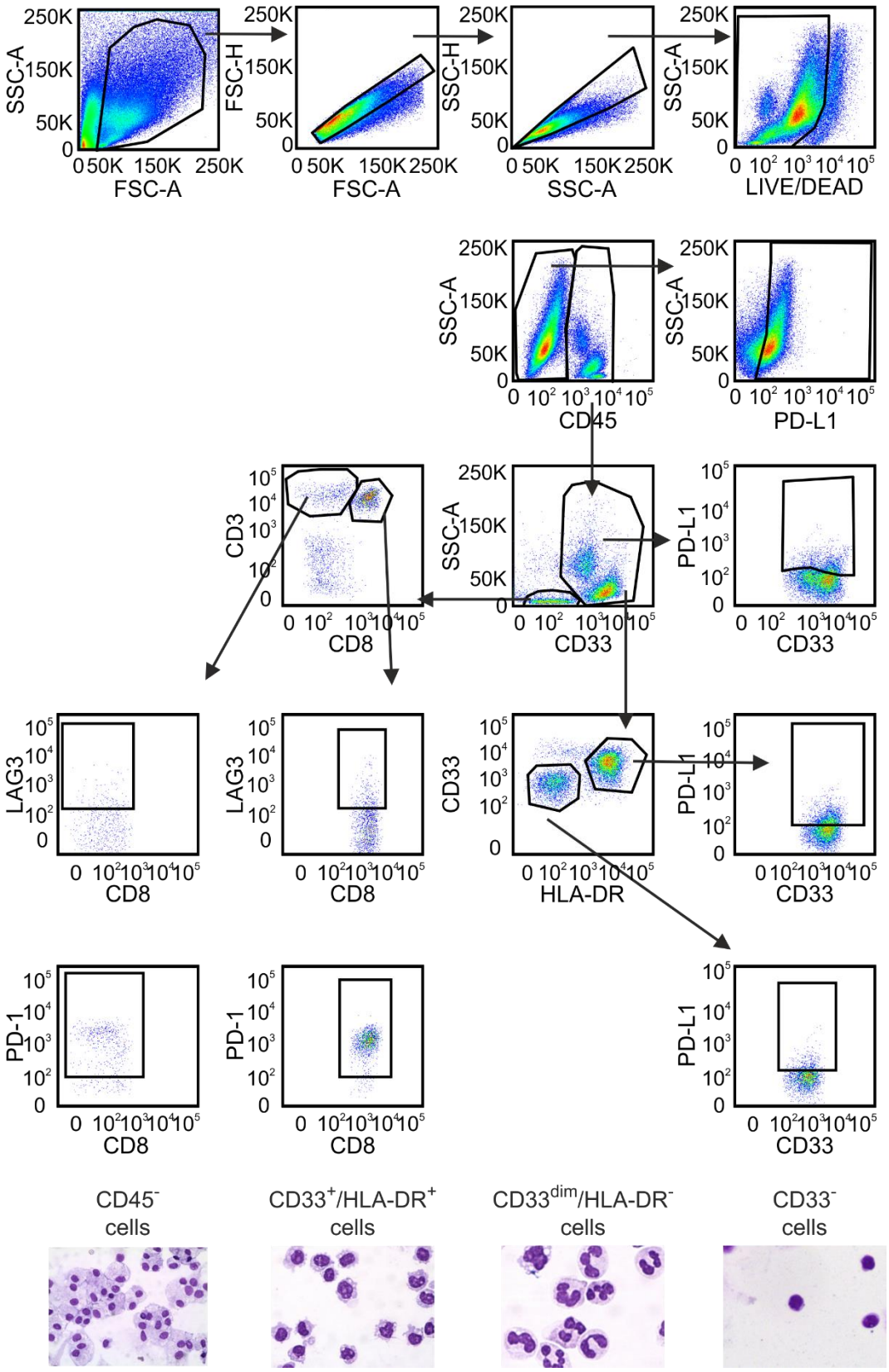

SSC-A
FSC-A
FSC-H
SSC-H
LIVE/DEAD
CD45
PD-L1
CD3
CD8
CD33
LAG3
HLA-DR
PD-1
CD45- cells
CD33+/HLA-DR+ cells
CD33dim/HLA-DR- cells
CD33- cells

**Fig. 9 Gating strategy used to analyze myeloid and T cells infiltrating tumor biopsies of MNG patients**. Among $CD33^+$ myeloid cells, two main subsets were distinguished on the basis of HLA-DR level: $CD33^+$/HLA-$DR^+$ and $CD33^{dim}$/HLA-$DR^-$. PD-L1 expression was evaluated on $CD45^-$ cells and on myeloid cell subsets, setting the gates on FMO controls. Among $CD33^-$ leukocytes, $CD3^+/CD8^-$ and $CD3^+/CD8^+$ lymphocytes were gated and the expression of LAG-3 and PD-1 was evaluated, based on FMO controls. Morphological analysis was performed by cytospins of $CD45^-$, $CD33^+$/HLA-$DR^+$, $CD33^{dim}$/HLA-$DR^-$ and $CD33^-$ cells stained with MGG.

Interestingly, we found that in grade II meningiomas there is a significantly higher presence of macrophages, as compared to grade I tumors (Fig. 10).

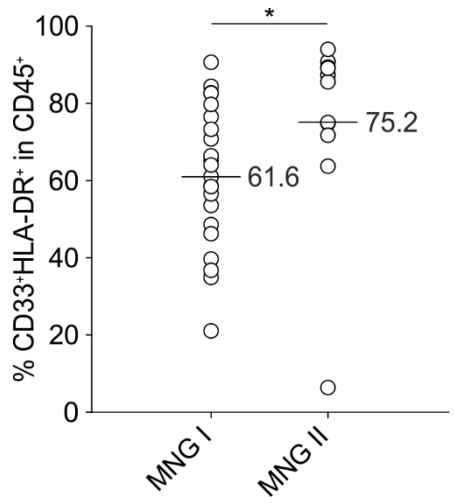
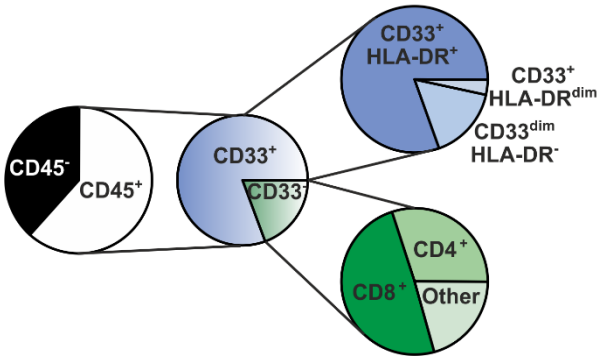



**Fig. 10 Distribution of macrophage population in MNG patients.**
The percentage of expression of macrophages $CD33^+$/HLA-$DR^+$ was evaluated in grade I (N=23) and grade II (N=11) meningioma patients. Mann-Whitney U test was performed. Asterisks denote significant P values: *P<0.05.

We also analyzed the lymphoid infiltrate, by gating on $CD33^-$ cells that phenotypically and morphologically corresponds to lymphocytes, and we used CD3 and CD8 markers to identify $CD3^+CD8^-$ (corresponding mainly to $CD4^+$ cells) and $CD3^+CD8^+$ T cells. $CD33^-$ cells constitute the 19.2±14.0% of leukocytes present at tumor site, in which $CD3^+CD8^-$ T cells (29.9±14.3%) and $CD3^+CD8^+$ T cells (49.0±14.7%), can be distinguished; these populations represent the 6.5±8.0 and 8.7±5.1% of total leukocytes, respectively (Fig. 11).

A




B

| Populations | Mean(%) ± SD |
|---|---|
| ❖ CD45- | 37.1 ± 24.9 |
| ❖ CD45+ | 59.5 ± 25.6 |
| ➢ CD33+ | 79.8 ± 14.6 |
| • CD33+/HLA-DR+ | 80.4 ± 20.6 |
| • CD33dim/HLA-DR- | 16.2 ± 17.6 |
| • CD33+/HLA-DRdim | 3.4 ± 4.2 |
| ➢ CD33- | 19.2 ± 14.0 |
| • CD3+/CD8-(CD4+) | 29.9 ± 14.3 |
| • CD3+/CD8+ | 49.0 ± 14.7 |
| • Other lymphocytes | 20.6 ± 9.8 |

**Fig. 11 Characterization of myeloid and lymphoid cells infiltrating the MNG tissue.**

A). Representation of the levels of different myeloid and lymphoid cells infiltrating tumor biopsies of meningioma patients. B). Mean and standard deviation (SD) of the percentage of different cell subsets present among tumor cells.

### 4.4 Characterization of tumor-infiltrating leukocytes in primary brain tumors

To characterize the composition of leukocyte infiltrate in GBM tissues at the time of surgery, we used a similar approach to that described for MNG patient.

We noticed a recurrent and important presence of infiltrating leukocytes in GBM (median=40.5%; Q1-Q3: 31.0-64.1%). The leukocyte population was mostly composed by $CD33^+$ myeloid cells (median=86.7%; Q1-Q3: 81.4-91.3%), the majority of which was characterized by macrophages ($CD33^+/HLA\text{-}DR^+$ median= 67.1%; Q1-Q3: 52.1-80.5%) and to a lesser extent by PMNs ($CD33^{dim}/HLA\text{-}DR^-$ median= 9.8%; Q1-Q3: 4.9-26.7%). Inside the $CD45^+$ population, we analyzed the presence of lymphocytes, and found only a minority of $CD3^+$ T cells (median=6.9%; Q1-Q3: 4.3-11.1%) almost equally divided in $CD3^+CD8^-$ (median= 3.2% of total leukocytes; Q1-Q3: 2.1-5.2%) and in $CD3^+CD8^+$ (median= 2.6% of total leukocytes; Q1-Q3: 1.6-4.2%) (Fig. 12).

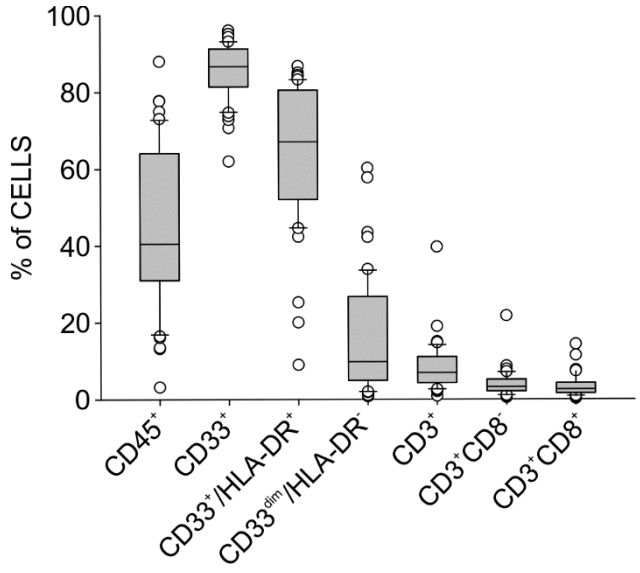



**Fig. 12 Composition of the tumor-infiltrating leukocytes in GBM patients.**

Box Plot represents the median, the 25$^{th}$ and 75$^{th}$ percentile of the frequency of myeloid and lymphoid populations in GBM biopsies. Whiskers extend to 1.5 inter-quartile range and outliers are shown by dots (N=51, for CD45$^+$, CD33$^+$, CD33$^+$/HLA-DR$^+$, CD33$^{dim}$/HLA-DR$^-$ cells; N=46 for CD3$^+$, CD4$^+$, CD8$^+$ cells). The gating strategy for the analysis of the GBM immune infiltrate was the same used for MNG samples. The percentage of CD45+ refer to the live cell proportion obtained after tumor dissociation while all the other cell subsets refer to the percentages of CD45$^+$ cells.

To deepen the analysis of the tumor microenvironment, we focused our attention on the expression of molecules involved in immunoregulation. We thus analyzed the presence on tumor cells and on myeloid subsets of programmed death-ligand 1 (PD-L1), the ligand of checkpoint PD-1, and found, in both tumors, that it was expressed at high levels among tumor cells (evaluated as CD45$^-$; median: 66.8%; Q1-Q3: 46.0-80.9% for GBM Fig. 13A, and median: 77.3%; Q1-Q3: 40.1-93.9% for MNG, Fig. 13B). Whereas in macrophages (33$^+$DR$^+$ median: 16.6%; Q1-Q3: 4.0-44.5% for GBM Fig. 13A, and median: 19.3%; Q1-Q3: 4.4-54.2% for MNG, Fig. 13B) and PMNs (CD33$^{dim}$HLA-DR$^-$ median: 8.7%; Q1-Q3: 2.6-17.3% for GBM fig. 13A, and median: 17.2%; Q1-Q3: 7.4-44.6% for MNG, Fig. 13B) the level of expression was lower and quite comparable, although the macrophages of some patients achieved the same PD-L1 levels present in tumor cells.

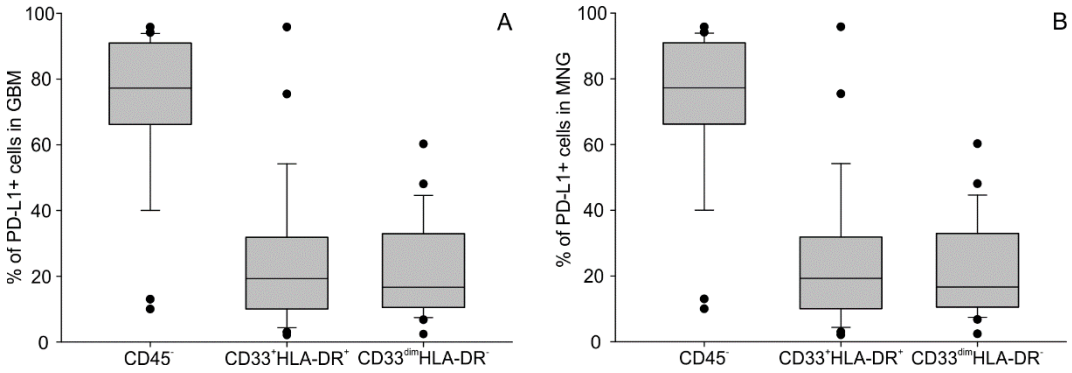



**Fig. 13 Expression of PD-L1 on cells in the microenvironment of primary brain tumors.**
Box plot reporting the median, the 25th and 75th percentile of PD-L1 expression in $CD33^+/HLA\text{-}DR^+$ (N=50 for GBM, 13A, and N=28 for MNG, 13B), $CD33^{dim}/HLA\text{-}DR^-$ (N= 50 for GBM, 13A, and N=28 for MNG, 13B), and $CD45^-$ cells (N=46 for GBM, 13A, and N=28 for MNG, 13B).

On the basis of these results, we investigated whether the receptor of PD-L1, programmed cell death protein 1 (PD-1), was expressed on infiltrating T cells, and observed, in both tumors, a high expression of PD-1 in $CD4^+$ T cells (evaluated as $CD3^+CD8^-$, mean 79.0% for GBM and 77.8% for MNG) and $CD3^+CD8^+$ (mean 80.4% for GBM and 87.4% for MNG). Furthermore, only in MNG patients, we noticed a significantly higher expression of PD-1 by $CD8^+$ T cells (Fig. 14).
Since PD-L1 is expressed on myeloid and tumor cells and PD-1 on T lymphocytes, we performed a statistical analysis to investigate whether there was a correlation between the expression of the two markers, but no statistical significance was reached in both tumor types for all the populations analyzed (data not show).

We also evaluated another marker involved in immune regulation, lymphocyte-activation gene 3 (LAG-3) (fig. 14C and D), which is implicated in the exhaustion of T cells. Our data indicate that, in both tumors, LAG-3 was expressed in T cell populations infiltrating meningioma biopsies (mean in $CD3^+CD8^-$: 10.2% for GBM and 19.3% for MNG; mean in $CD3^+CD8^+$: 11.4% for GBM and 19.5% for MNG) and the levels of expression were comparable to each other.

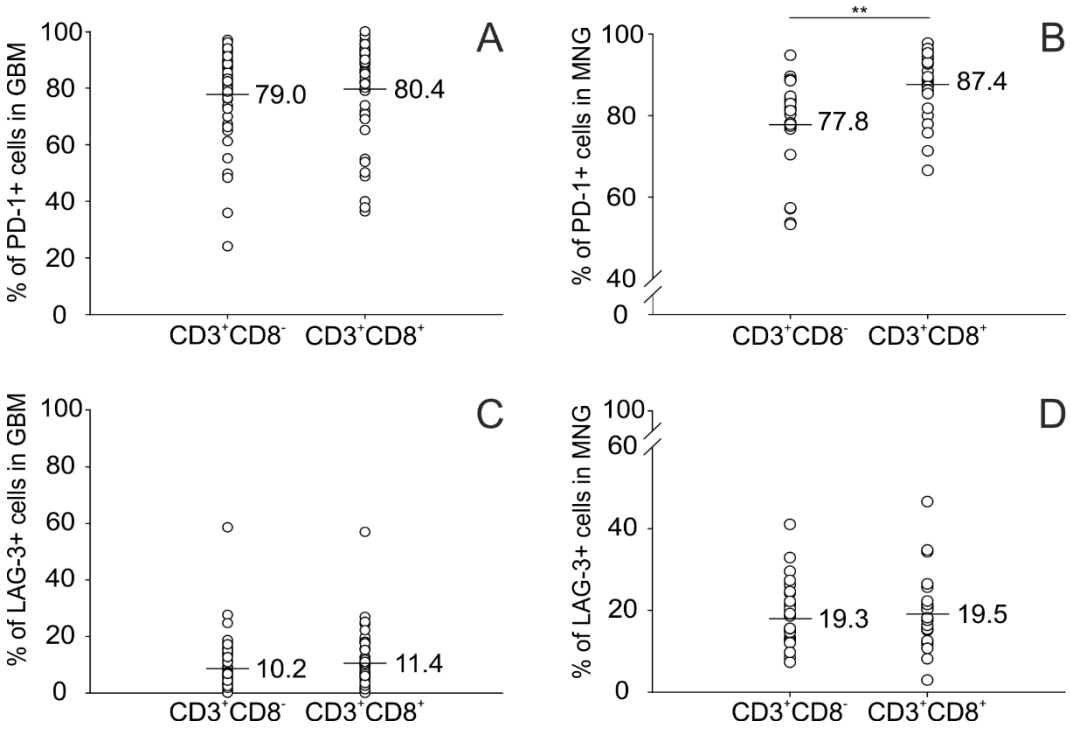



**Fig. 14 Expression of markers PD1 and LAG-3 on cells infiltrating GBM tissue.**

Percentages of expression of the markers PD-1 (A and B panels) and LAG-3 (C and D panels) were calculated in the $CD3^+/CD8^-$ and $CD3^+/CD8^+$ T cell subsets (N=46). Mann-Whitney U test was performed. Asterisks denote significant P values: **P<0.01.

In conclusion, meningiomas and glioblastomas show a large and significant presence of tumor-infiltrating leukocytes, characterized mainly by myeloid cells, and by a small but constant presence of lymphocytes. Moreover, PD1 and PD-L1 are highly expressed on different cells of the microenvironment, thus suggesting an ongoing impairment of the immune response.

### 4.5 Expansion of MDSCs in primary brain tumors

Given the expression of immune-regulatory molecules on tumor-infiltrating leukocytes, we evaluated also the presence at the tumor site of MDSC subsets. For this analysis, we used the same phenotypic strategy used to detect blood MDSC, although it should be taken into account that at the tumor site immature myeloid cells might undergo to a phenotypic transition and bear a slightly changed phenotype. After tissue dissociation, cells were stained with 2 panels containing 9 different markers, and MDSC subsets were analyzed using the gating strategy

shown in Fig. 15. Among the CD15$^-$ cells, we recognized MDSC 1, defined as CD14$^+$/IL4R$^+$, and MDSC 4 identified as CD14$^+$/HLA-DR$^{low/-}$. MDSC 2 were gated among CD15$^+$ cells as CD15$^+$/IL4R$^+$ cells and MDSC 3 were discriminated from the whole leukocyte population as Lin$^-$/HLA-DR$^-$ cells expressing CD33 and CD11b.

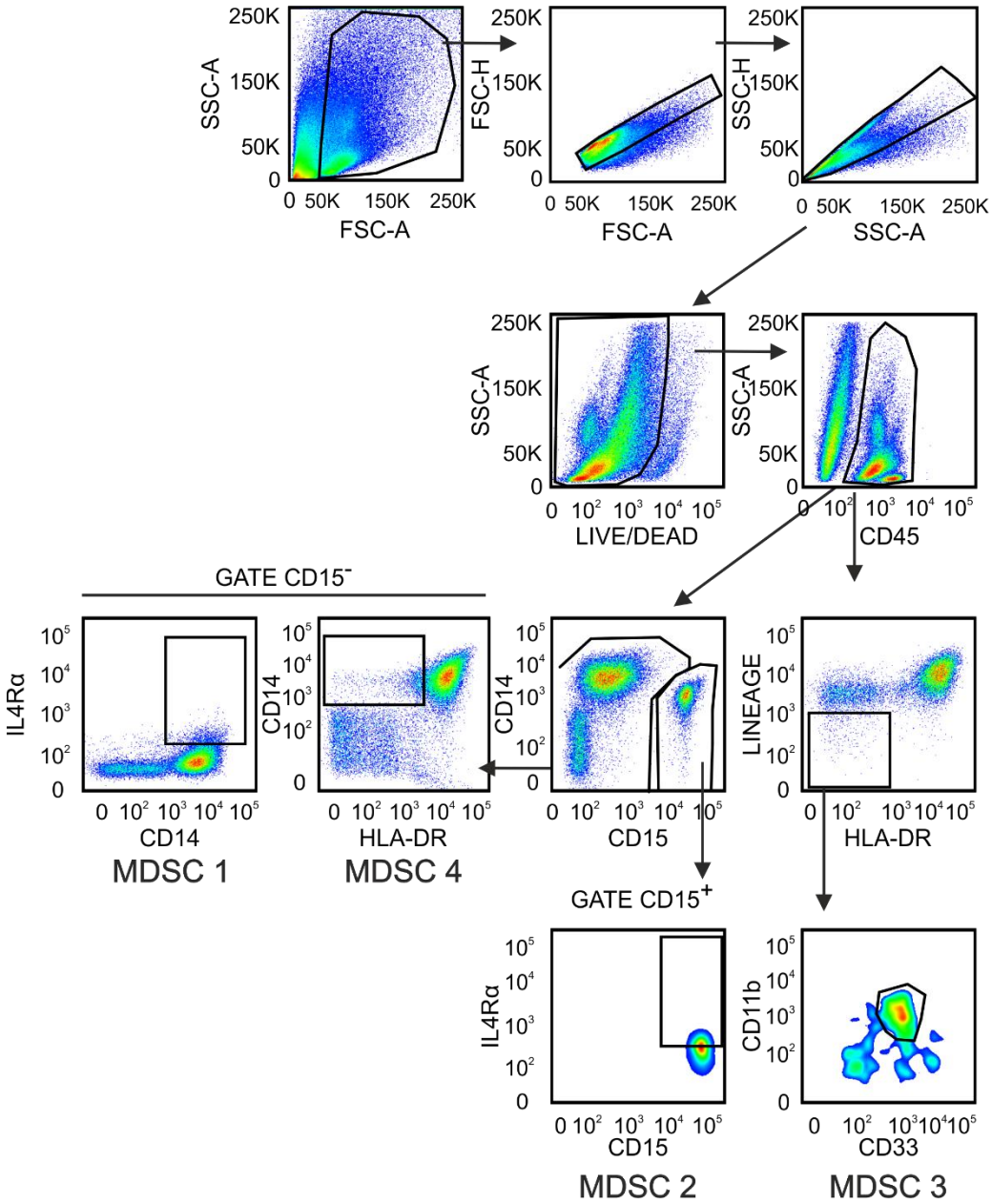


**Fig. 15 Analysis of MDSC subsets present at the tumor site**. Gating strategy for the definition of MDSC subsets in MNG and GBM tumors. Following morphological gate and doublets exclusion, live cells were gated as LIVE/DEAD$^-$ and leukocytes were defined by CD45 marker. MDSC 1 and 4 were identified among CD15$^-$ leukocytes as CD14$^+$/IL4Rα$^+$ (MDSC 1) or CD14$^+$/HLA-DR$^{low/-}$ (MDSC 4). MDSC 2 were defined as CD15$^+$ leukocytes expressing IL4Rα, while MDSC 3 were gated as Lin$^-$/HLA-DR$^-$ cells expressing CD33 and CD11b markers. Gates for IL4Rα and HLA-DR were set on FMO controls.

This analysis revealed that MDSC levels are detectable in the tumor infiltrate of both tumors, and we could appreciate that MDSC 4 show the highest expansion in GBM patients (Fig. 16D), and its expression is significantly higher compared to MNG patients. We could also find a significant increase of MDSC 2 in GBM as compared to MNG (Fig. 16B), while MDSC 1 and 3 are less abundant (Fig. 16A and 16C, respectively) and the level of expression is equivalent between the two tumor types.

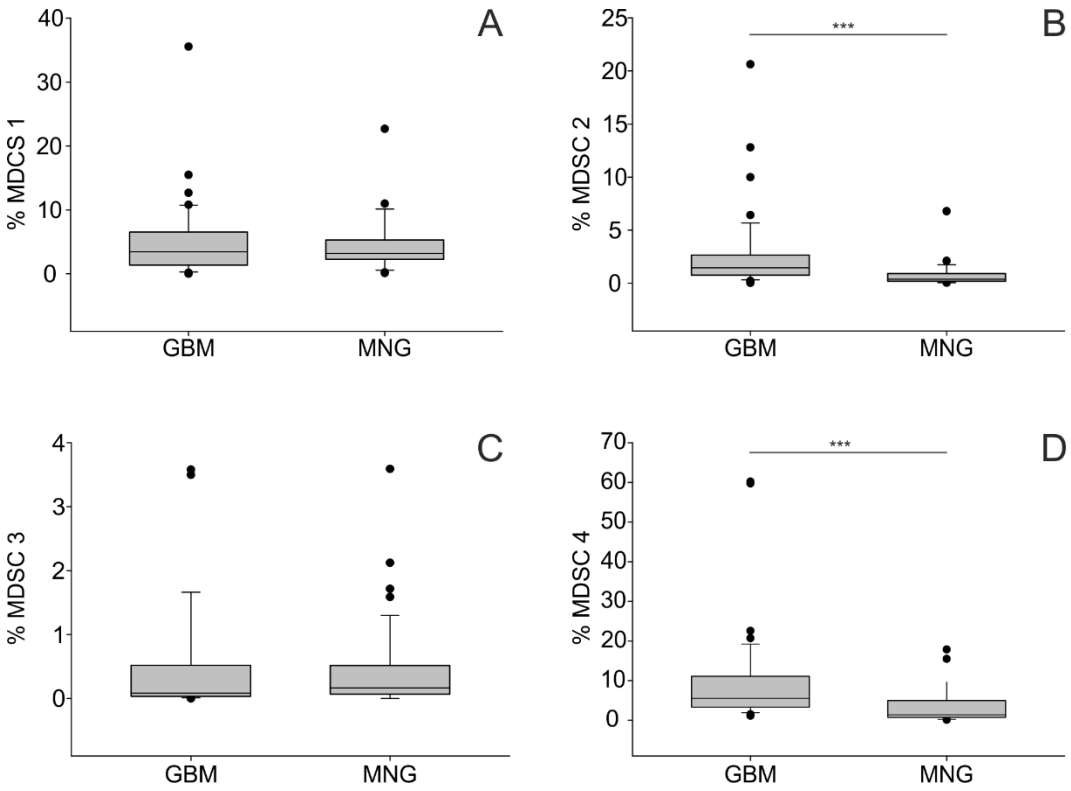



**Fig. 16 Levels of MDSC subsets in tumor biopsies of MNG and GBM patients.**
Box plot reporting the median, the 25$^{th}$ and 75$^{th}$ percentile of MDSC subsets expression in MNG (N=24 for MDSC 1 and 2; N=28 for MDSC 3; N=26 for MDSC 4) and GBM biopsies (N=46 for MDSC 1; N=45 for MDSC 2; N=44 for MDSC 3; N=48 for MDSC 4). Mann-Whitney U test was performed. Asterisks denote significant P values: ***P<0.001.

### 4.6 Dissecting the presence of macrophages in GBM microenvironment

Our phenotypic characterization showed an expansion of the macrophages in both tumors considered. We reasoned that the brain contains several myeloid populations, located at distinct sites. In fact, under physiological conditions in the parenchyma the only myeloid cells are resident microglial cells, and macrophages are found in the outer boundaries of the brain, such as the choroid plexus, perivascular spaces, and in the meninges (Prinz and Priller 2014) (Li and Barres 2018). However, in a brain with a growing tumor, the presence of BMDM has been clearly demonstrated, especially in mouse models (Bowman, Klemm et al. 2016). Until recently, the precise identification of human microglial cells from BMDM was limited to morphological evaluation coupled to immunohistochemical myeloid markers, or to subtle differences in staining intensity of myeloid markers by flow cytometry, due to the lack of a marker differentially expressed on the two cell types. However, recently the addition of CD49D marker has been proposed to discriminate microglia from BMDM (Bowman, Klemm et al. 2016) (Zong 2017).

We thus set out to discriminate in GBM tissues resident from blood-derived macrophages, by using CD49D in combination with CD45, CD33 and HLA-DR markers. This analysis showed the presence of two main macrophage subsets, identified as $CD45^+/CD33^+/HLA\text{-}DR^+/CD49D^+$, corresponding to BMDM and $CD45^+/CD33^+/HLA\text{-}DR^+/CD49D^-$, corresponding to MG cells. Afterwards, we sorted these two myeloid cell populations in order to tests their functional activity, and prepared them for cytospin examination. The morphological evaluation of these subsets indicates that these two populations have distinct characteristics; in fact, $CD49D^+$ BMDMs (Fig. 17A) are larger cells, with an abundant and vacuolated cytoplasm, and smaller nucleus-to-cytoplasm ratio, corresponding to a typical morphology of tissue macrophages, while resident MG $CD49D^-$ cells are smaller cell with a larger nucleus-to-cytoplasm ratio, indicating a different embryonal origin (Fig. 17B). We confirmed these morphological differences by flow cytometry analysis, in which BMDM displaying a significantly higher SSC than MG (Fig. 17).

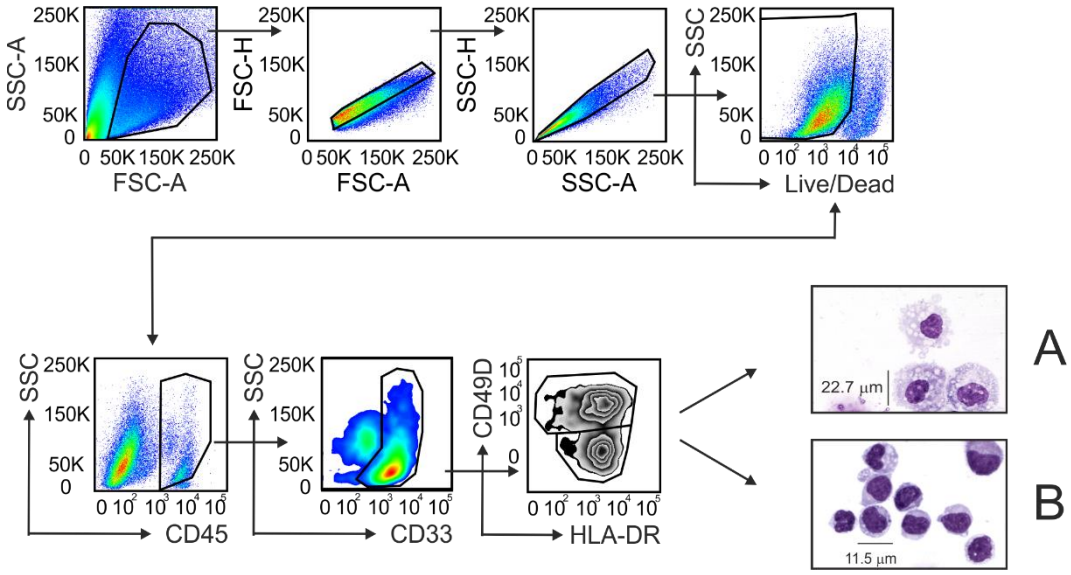



**Fig. 17 Phenotypic and morphological examination of MG and of BMDM in GBM tissues.** After morphological gate and doublets exclusion, live cells were gated as LIVE/DEAD$^{-}$. A gate on CD45$^{+}$ cells was drawn, excluding lymphocytes (CD45$^{high}$/SSC$^{low}$ cells) and then macrophages were identified as CD33$^{high}$. CD49D and HLA-DR markers allow the phenotypical identification of BMDM (CD49D$^{+}$/HLA-DR$^{+}$) (A) and of MG (CD49D$^{-}$/HLA-DR$^{+}$) (B) in GBM biopsies. Morphological analysis was performed by cytospins of CD49D$^{+}$/HLA-DR$^{+}$ and CD49D$^{-}$/HLA-DR$^{+}$ cells stained with MGG.

We therefore investigated the distribution of macrophage subsets in 27 GBM biopsies and observed a significant higher presence of BMDM as compared to MG (Fig. 18), thus highlighting that the main macrophage population present at tumor site originates from the bone marrow and is actively recruited in GBM.

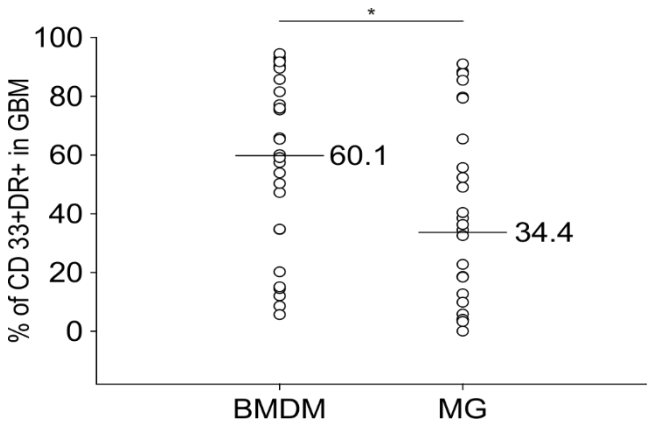


**Fig. 18 MG and BMDM levels among myeloid cells present in GBM biopsies.**
Percentages of expression of macrophages were calculated among the $CD45^+/CD33^+/HLA\text{-}DR^+$ cell population (N=27). Mann-Whitney U test was performed. Asterisks denote significant P values: *P<0.05.

### 4.7 Functional activity of macrophages isolated from tumor biopsies and peripheral blood in brain cancer patients

Although macrophages have the potential to attack and eliminate tumor cells, those found in the tumor tissue often exhibit many pro-tumoral features, and interfere with the function and proliferation of immune effectors. We thus decided to test the immune suppressive activity of macrophages present in tumor biopsies, to determine their role in the tumor microenvironment. To this aim, we sorted live $CD33^+/HLA\text{-}DR^+$ cells from MNG biopsies, and $CD45^+/CD49D^+/HLA\text{-}DR^+$ cells (BMDM) or $CD45^+/CD49D^-/HLA\text{-}DR^+$ cells (MG) from GBM patients and tested their ability to interfere with the proliferation of allogeneic T cells after anti-CD3/CD28 stimulation. From MNG samples we sorted total $CD33^+DR^+$ cells, without a further characterization, because we had not investigated in detail the macrophage subsets present in the meninges.

T cell suppression rate was calculated with two different methods that take into account two aspects of immunosuppression. In fact, in some samples immunosuppression is detected as a reduction in T cell number and we define it as “quantitative” immunosuppression (Fig. 19B)., while in other cases CellTrace profile is altered and a lower number of cell cycles is detected. We thus define this as “qualitative” immunosuppression (Fig. 19A).

Results from these experiments are shown in Fig. 19C, and reveal that $CD33^+HLA\text{-}DR^+$ cells isolated from 6 MNG patients (dark grey histograms) and BMDMs isolated from 7 GBM samples (pale grey histogram) exerted a strong immunosuppression on the proliferation of T cells, while MG isolated from 4 glioblastoma samples (black histogram) showed a lower immunosuppressive activity.

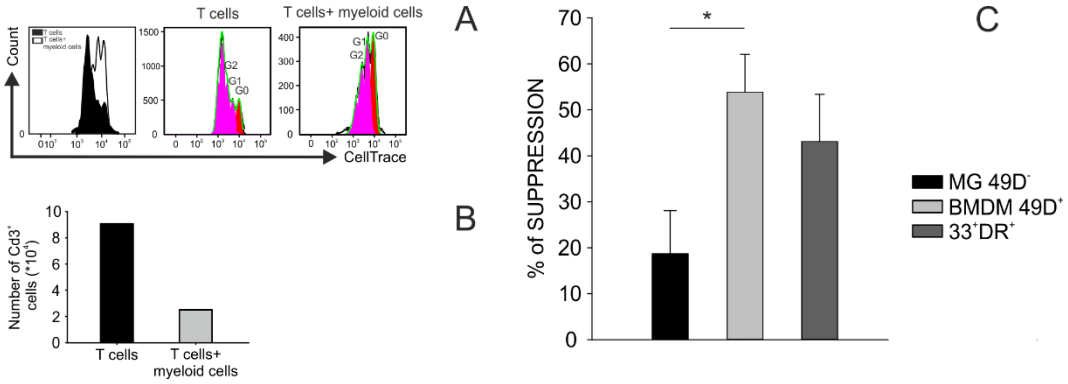



**Fig. 19 Functional analysis of myeloid cells isolated from MNG and GBM tumor tissue.**
Immunosuppressive activity of macrophages in primary brain tumors. Panel A) shows a representative example of qualitative suppression, while panel B) shows a representative example of quantitative suppression. C) Immunosuppressive activity of $CD33^{+}/HLADR^{+}$ (N=6), BMDM (N=7) and MG (N=4) cells isolated from biopsies by immunomagnetic or FACS sorting. Dark gray histogram refers to $CD33^{+}HLA\text{-}DR^{+}$ from MNG tissues, while pale gray histogram shows suppression by BMDMs ($CD49^{+}$) and black histogram by MGs ($CD49^{-}$) of GBM patients. Mann-Whitney U test was performed. Asterisks denote significant P values: *P<0.05.

Altogether, these results confirm the existence of a strong immune suppression in the TME mediated by macrophages infiltrating both MNG and GBM tissue, and a lower level of suppression exerted by resident MG cells.

# 5. DISCUSSION

Increasing evidence shows that the cross-talk among tumor cells, immune cells and matrix components is mediated by a complex network of interactions that dynamically modulate the tumor microenvironment. Such interactions are able to drive either tumor expansion or regression, since many infiltrating immune cells possess a dual function in the tumor milieu. A new target of investigation is the role of the immune system since systemic and local immune suppression plays a relevant role in tumor progression, and in this respect, many efforts are ongoing trying to re-educate and reinvigorate the immune cells in order to convert the immune system from detrimental to protective.

Our group demonstrated that in primary brain tumors there is a significant presence of leukocytes, many of which express molecules involved in immunoregulation, like PD-L1, PD-1 and LAG-3. Of note, the vast majority of infiltrating leukocytes are myeloid cells that, beforehand, could have a positive or a negative impact on tumor progression. Our study evaluated in detail the composition and functional activity of myeloid at the tumor site, and found that macrophages are the main component of meningiomas and glioblastomas. We also evaluated the presence of several subset of MDSCs also in the tumor site, but we found a limited amount of them. In fact, an issue that is not yet resolved is whether MDSCs, once they reach the tumor site, maintain the same phenotype observed in the circulation, or, given their inherent plasticity, can adapt in response to the microenvironmental stimulation and be assimilated to tissue macrophages. Regarding GBM, we were able to define the exact composition of blood-derived and resident macrophages, thanks to the phenotypic definition of the two cell types. This led us to the *ex-vivo* purification of both myeloid cells from untreated patients, and to test their activity on activated T cells, leading to the conclusion that blood-derived macrophages possess a strong immune suppressive activity at the tumor site, while resident MG cells show a lower or negligible activity. Therefore, we hypothesize that, despite both macrophage populations (MG and BMDM) share the same immunosuppressive microenvironment, the BMDMs are more affected by the signals carried by TME, that induce their immune suppression activity. Of note, also benign MNGs show a large infiltrate of immune suppressive macrophages, even if greater in grade II MNGs than grade I MNGs, a feature accumulating the two tumors (Fig. 11 and Fig. 12). Although a direct comparison of the immune suppressive activity is hard to perform, our results suggest that the extent of

suppression showed by BMDM separated from GBM tissues is higher than that exerted by MNG-derived macrophages. In addition, by dividing myeloid cells isolated from grade I and grade II MNG patients we observed a trend towards increased immunosuppression in grade II MNG patients, although not statistically significant (data not shown). These results indicate that any immunotherapeutic approach toward these tumors should consider the hostile microenvironment for the activity of the immune system.

Our results reinforce the notion that BMDM targeting could be an effective therapeutic instrument for the treatment of primary brain tumors. Recent studies have described an important role for colony-stimulating factor-1 receptor (CSF-1R) in brain suppressive macrophages biology, such that inhibition of CSF-1R either depletes (Coniglio, Eugenin et al. 2012) or depolarizes (Pyonteck, Akkari et al. 2013), depending on the preclinical model tested, leading to reduced glioma growth and invasion (Quail, Bowman et al. 2016). In light of their plasticity, increasing advances are ongoing to develop strategies that re-educate macrophages to adopt anti-tumor phenotypes in cancer, including brain tumors, which are likely to be more efficacious than depleting all suppressive macrophages populations (Bowman and Joyce 2014) (Quail and Joyce 2013). Whether CSF-1R inhibitors ultimately represent the most effective means to achieve this goal remains to be seen, as different preclinical glioma models have shown that acquired resistance to CSF-1R inhibition ultimately develops in approximately half of the treated animals (Quail, Bowman et al. 2016).

In this study, we demonstrated the presence of an alteration of myeloid compartment in both MNG and GBM patients. Indeed, we confirmed an expansion of circulating MDSCs and a decrease of circulating intermediate monocytes, that showing an increase of their surface of CCR2 expression, which suggest an active process of monocyte recruitment induced by the TME. It thus appears that circulating myeloid represent the source sustaining myeloid accumulation in the lesion, accompanying tumor growth. Therefore, blocking macrophage recruitment to the tumor might be a therapeutic strategy to limit tumor growth. A new study has used human TCGA database in order to stratify the GBM patients on the basis on the expression of CCL2 and it has divided the patients into high and low CCL2 cohorts, finding that GBM patients with a low CCL2 expression survived

significantly longer than those with a high CCL2 expression (Chen and Hambardzumyan 2018). These findings raise the question as to whether reducing monocyte infiltration by targeting the CCL2–CCR2 axis may be a viable option for treating GBM. To address this question, this study showed that genetically interrupting the CCL2–CCR2 axis prolonged the survival of GBM-bearing mice, in agreement with previous pharmacological studies (Zhu, Fujita et al. 2011) (Chang, Miska et al. 2016). However, in contrast to the promising preclinical studies, neutralizing monoclonal antibodies against CCL2 administered to patients with metastatic, solid tumors did not produce favorable outcomes (Chen and Hambardzumyan 2018).

Primary brain tumors develop numerous immunosuppressive mechanisms to resist antitumor immune attack, such as the production of immunosuppressive factor, like ARG-1 (Elgert, Alleva et al. 1998) (Chouaib, Asselin-Paturel et al. 1997) (Chen and Hambardzumyan 2018) (Nduom, Weller et al. 2015). Accordingly, our data indicate an increased production and functionality of the arginase enzyme in both patients with GBM and MNG. Moreover, our data showed a significant increase in the enzymatic activity of ARG-1 at pH 7.3 in the two types of tumor compared to alkaline pH albeit in GBM patients the functional activity of ARG-1 is greater than MNG patients. A possible explanation for this phenomenon is that GBM alters the regulation of ARG, thus enhancing its immune regulatory activity in malignant GBM, compared to benign MNG. These results open a new area of investigation, implicating the regulation of the activity of the immune regulatory enzyme ARG-1 in cancer patients. It has been extensively reported that ARG-1 catalytic activity largely depends on pH. Indeed, the curves of pH dependency for this enzyme show that ARG-1 enzymatic activity is maximal in a strong alkaline environment (pH 9.5–10.5) and negligible at neutral pH (Folley and Greenbaum 1948) (Ikemoto, Tabata et al. 1990), although the enzyme acts under physiological conditions. In recent years, a study on PMN from HD has clarified this phenomenon (Rotondo, Bertolotto et al. 2011). This work has shown that ARG-1 released by PMNs is inactive at physiological pH, unless activated by proteolytic factors. In fact, the enzymes stored inside the intracellular granules of the PMNs are usually kept in the form of inactive proenzymes that are activated by proteolytic cleavage after release (Gullberg, Bengtsson et al. 1999). Therefore, the native form of this enzyme is active at pH values that are never reached within our body. The necessity for ARG-

1 to be cleaved in order to exert its function at physiological pH is indicative of the fact that there may be a control mechanism able to prevent accidental activation of the enzyme and subsequent immune suppression. Therefore, the behavior of ARG-1 in our samples seems to be congruent with the results of this study, because factors released by the tumor microenvironment can induce the proteolytic cleavage of this enzyme with the consequent activation of ARG-1 at physiological pH.

In the last decades, immunotherapy has emerged as a potent alternative for cancer treatment. Checkpoint inhibitors, like anti-PD-1 and anti-CTLA-4 antibodies, as well as adoptive T cell therapy have been approved for use in an increasing type of cancers, including melanoma, non-small-cell lung cancers, bladder, kidney and Hodgkin lymphoma (Khalil, Smith et al. 2016).
Anti–PD-1 blockade has been shown to improve survival in murine glioma model systems in combination with radiotherapy (Zeng, See et al. 2013) (Reardon, Gokhale et al. 2016). Additionally, in a murine model of intracerebral glioma, combinatorial therapy of IDO, CTLA-4, and PD-L1 was curative in a marked number of mice (Wainwright, Chang et al. 2014). Besides, an alternative approach for inhibiting Tregs is the use of the anti-IL2Rα antibody (daclizumab), which has been shown to decrease Treg numbers and increase the ratio of effector T cells to Tregs in patients undergoing standard-of-care treatment with temozolomide (Sampson, Schmittling et al. 2012). Cumulatively, these data provided a sufficient rationale for the use of immune checkpoint inhibitors, and a variety of clinical trials are currently under way using these strategies in GBM patients (Sampson, Maus et al. 2017). Furthermore, iIpilimumab and pembrolizumab have been shown to have acceptable safety and some efficacy in patients with metastatic brain metastasis from melanoma or non–small-cell lung cancer (Goldberg, Gettinger et al. 2016) (Margolin, Ernstoff et al. 2012). However, despite the checkpoint inhibitors also showed a modest but significant clinical activity in a subgroup of patients in the early stages of the experimentation (Omuro, Vlahovic et al. 2018), monotherapy with anti-PD-1 or combinational therapy with anti-PD-1 and anti-CTLA-4 antibodies for the treatment of recurrent GBM recently failed in phase III clinical trial (Filley, Henriquez et al. 2017). This failure implies that the simple blockade of immune checkpoints may not restore the functions of T cells, which may be intrinsically impaired or exhausted. These result set the ground for several

considerations, also including the notion that GBM should not be viewed as a single entity, but rather as a tumor with at least 4 molecular subtypes, which may not respond equally to a given therapy (Verhaak, Hoadley et al. 2010). Indeed, about 8% of all patients in this trial responded well to Nivolumab (Filley, Henriquez et al. 2017). However, a phase II clinical trials involving two anti-PD-1 antibodies; Durvalumab (NCT02336165) and Pembrolizumab (NCT02337491) are currently underway in primary GBM patients. Moreover, a randomized Phase III trial testing intravenous administration of Nivolumab (NCT02017717), an anti-PD-1 antibody, in recurrent GBM patients, alone and in combination with Bevacizumab or the anti-CTLA-4 drug Ipilimumab, is due for completion in 2018 (Tivnan, Heilinger et al. 2017). Considering the immunological landscape in brain tumors, it becomes clear that exploiting the immune system for therapeutic purposes may require therapeutic combinations aiming at blocking major GBM-derived inhibitory hubs as well as a re-invigoration of innate and adaptive immune reactions by additional measures such as vaccination. Actually, a variety of vaccination strategies have demonstrated encouraging preliminary results (Bloch, Crane et al. 2014) (Hunn, Bauer et al. 2015) (Mitchell, Batich et al. 2015) (Van Gool 2015) (Sampson, Aldape et al. 2011) (Sampson, Schmittling et al. 2012). These vaccines have targeted normal or overexpressed tumor proteins within the tumor (Bloch, Crane et al. 2014), proteins with specific amino acid changes that resulted from tumor-specific neo-epitopes, or viral antigens (Sampson, Aldape et al. 2011) (Sampson, Heimberger et al. 2010).

It is currently being explored a therapeutic approach involving chimeric antigen receptor (CAR) cell technology, in which naïve T cells are transduced with a construct expressing a receptor for a specific antigen (Sampson, Maus et al. 2017). T cells bearing a CAR can be activated by this antigen in the absence of any co-stimulation, who should ideally be expressed exclusively by tumor cells to avoid any collateral damage to normal tissues. Currently, phase I/II clinical trials are exploiting CAR T cells targeting the glioma-specific antigens human epidermal growth factor receptor 2/neu (EGFRvIII) and interleukin-13 receptor subunit alpha-2 (IL-13Rα2), which have demonstrated promising results in preclinical models (Sampson, Choi et al. 2014) (Krebs, Chow et al. 2014) (Brown, Badie et al. 2015). Brown et al recently reported a striking response in a patient with a poor prognosis of multifocal GBM after multiple intraventricular infusions of IL-13Rα2 CAR T cells. This raises the possibility that local delivery of CAR T cells may enhance response

in GBM and that the route of administration is important (Brown, Alizadeh et al. 2016). It thus appears that, to identify new therapeutic targets of intervention for the treatment of primary brain tumors, it is essential to implement our knowledge on the complex immunological landscape of brain tumors.

# 6. ABBREVIATIONS

ADAM17: ADAM Metallopeptidase Domain 17

ALT: alternative telomere elongation pathway

APC: antigen presenting cell

ARG-1: arginase 1

B7-H1: B7 homolog 1

BBB: blood-brain barrier

BMDMs: bone marrow-derived macrophages

CCL2: C-C motif chemokine ligand 2

CCL22: C-C motif chemokine ligand 22

CCR2: C-C chemokine receptor type 2

CCR4: C-C chemokine receptor type 4

CD49D/ITGA4: integrin subunit alpha 4

CD95L: Fas ligand

CDKN2A: cyclin-dependent kinase inhibitor 2A

CNS: central nervous system

CSF: cerebrospinal fluid

CTL: cytotoxic T lymphocyte

CX3CL1: chemokine (C-X3-C motif) ligand 1

CX3CR1: CX3C chemokine receptor 1

CXCL10: C-X-C motif chemokine ligand 10

CXCL9: chemokine (C-X-C motif) ligand 9

DC: dendritic cell

ECM: extracellular matrix

EGF: epithelial growth factor

EGFR: epidermal growth factor receptor

e-MDSCs: early stage myeloid-derived suppressor cells

FBS: fetal bovine serum

FMO: fluorescence minus one

FoxP3: forkhead box P3

FSC: forward scatter

GBM: glioblastoma

GM-CSF: granulocyte-macrophage colony-stimulating factor

IDH1: isocitrate dehydrogenase 1

IDO: indoleamine-2,3-dioxygenase

IFN: interferon

IL-10: interleuchine 10

IL4Rα: α chain of interleukin 4 receptor

IMDM: Iscove's modified Dulbecco's medium

iNOS: inducible Nitric Oxide Synthase

JAM: junctionals adhesion molecule

LAG-3: lymphocyte-activation gene

mAb: monoclonal antibody

M-CSF: macrophage colony-stimulating factor

MDSCs: myeloid-derived suppressor cells

MG: microglia

MGMT: methyltransferase gene

MHC: major histocompatibility complex

MNG: meningioma

Mo-MDSCs: monocytic myeloid-derived suppressor cells

NF1: neurofibromatosis type 1

NF2: neurofibromatosis type 2

NK: natural killer

NKT: natural killer T cells

NO: nitric oxyde

norNOHA: Nv-hydroxy-nor-Arginine

OMS: organizzazione mondiale della sanità

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PD-1: programmed cell death-1

PDGFRA: platelet-derived growth factor receptor A

PD-L1: programmed death-ligand 1

PGE: prostaglandin E

PMN-MDSCs: polymorphonuclear myeloid-derived suppressor cells

PTEN: phosphatase and tensin homolog

RCAS1: receptor binding cancer antigen expressed on SiSo cells

RNS: reactive nitrogen species

ROS: reactive oxygen species

RTF: regeneration and tolerance factor

SAS: subarachnoid space

SSC: side scatter

TAMs: tumor-associated macrophages

TCGA: The Cancer Genome Atlas

TCR: T cell receptor

TERT: telomerase reverse transcriptase

TGF-β: transforming growth factor beta

Th: T helper

TIL: tumor infiltrating lymphocyte

TME: tumor microenvironment

Tmem119: transmembrane protein 119

TNF: tumor necrosis factor

TP53: tumor protein p53

Treg: regulatory T cells

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

WHO: World Health Organization

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