Tumors of the Central Nervous System

Central Nervous System (CNS) tumors are relatively rare, with the world incidence of primary brain tumors affecting 7 individuals in 100,000 inhabitants per year (Ostrom et al., 2015). They represent a vastly heterogeneous group of neoplasias originating from intracranial tissues and meninges. They vary according to their tissue of origin, location, dissemination pattern, clinical history, age of occurrence and diagnosis (Chen et al., 2016; Collins, 2004). CNS tumors are quite aggressive, and the percentage of a 5 years’ overall survival after diagnosis can be as low as 6.0% (Ostrom et al., 2015). The World Health Organization (WHO) recognizes over 120 forms of brain tumors (Louis et al., 2007, 2016) of which gliomas are the most frequent.

Gliomas are a heterogeneous group of primary neuroectodermal tumors, originating from glial cells – such as astrocytes and oligodendrocytes, or their progenitor cells. Previous classifications (Louis et al., 2007) subdivided gliomas in different grades of malignancy (grade I-IV) based mainly on histological and clinical features, along with tumor growing pattern. The current classification associates previous information with mutational data, specifically IDH1/2 mutations, whose alterations occur early in diffuse glioma tumorigenesis (both from astrocytic and oligodendrocytic origins) (Louis et al., 2016; Parsons et al., 2008). As a consequence, classification has become more complete, grouping prognostic markers and helping guide the treatment for gliomas that are biologically and genetically similar. Figure 1 displays the new classification.
Pilocytic astrocytoma

Grade I pilocytic astrocytomas (AGI) occur mainly in the cerebellum of children (Collins, 2004). In contrast to other gliomas, AGI are slow growing, circumscribed tumors. If surgical resection is possible, the prognosis is relatively good (Ichimura et al., 2004).

The genetic association of the neurofibromatosis type I gene, NF1, and AGI is well established (Reis and Tihan, 2015). NF1 is a transcriptional suppressor that negatively regulates RAS oncogene expression. When constitutively activated, RAS protein leads to the subsequent activation of the mTOR signaling pathway and of associated cell proliferation transcription factors.

The fusion between KIAA1549 and BRAF genes and a copy number gain of 1.9MB in chromosome 7q34, are also involved in the pathology of AGI. This is the most frequent genetic alteration in this type of tumor (Deshmukh et al., 2008; Pfister et al., 2008). The consequence of such fusion is the loss of the amino terminal domain of BRAF protein, constitutively activating its kinase activity and increasing cell proliferation.

Diffuse gliomas

Diffuse gliomas (classified as grade II-IV by WHO (Louis et al., 2016)) are infiltrative CNS tumors. Astrocytomas, oligodendrogliomas and glioblastomas (GBM) are included in this classification. Due to their invasive nature, complete surgical resection of these tumors is very difficult to achieve. The presence of residual tumor cells results in recurrence and malignant progression, albeit at different intervals. Part of lower grade tumors will either recur or progress to a GBM within months, while others will remain stable for years; the same is true for GBMs, with recurrence occurring at different rates (Cancer Genome Atlas Research Network et al., 2015;...
Ceccarelli et al., 2016; Foote et al., 2015; Kamoun et al., 2016). The determining factors for one behavior or another are not yet completely understood.

**Diffuse astrocytomas**

Grades II and III of astrocytoma (AGII and AGIII, respectively) occur mostly in adults. Their main feature, invasiveness, is what makes their prognosis worse, as that hampers complete surgical resection (Ichimura et al., 2015). Both tumors can progress to higher grade malignancies (Furnari et al., 2007), a process of high clinical relevance, since AGII patients present an overall survival (OS) of 7 years; this time is reduced by 50% in the case of AGIII and to less than 15 months in the case of grade IV tumors (glioblastomas, GBMs) (Binder et al., 2016).

Diffuse astrocytomas present molecular particular features identified in high performance genetic studies completed in the past few years (Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016). IDH1/2 mutations are present in most of the cases, and were recently listed as crucial for the diagnosis of AGII by the WHO (Louis et al., 2016). Mutations that inactivate the functions of both ATRX and TP53 are also frequent, with the first being essential to differentiate between astrocytic and oligodendrocytic tumors. Epigenetic alterations, such as methylation of CpG islands (G-CIMP) are present in 55% of cases (Ceccarelli et al., 2016).

**Oligodendrogliomas**

Oligodendrogliomas (OD) constitute less than 10% of all gliomas (Ostrom et al., 2015). ODs mainly occur in young adults and, although more delimited than astrocytomas, ODs also can infiltrate the surrounding normal brain tissue. In approximately 50% of the cases there is the presence of calcification, a feature that has been useful for clinical diagnosis (Wesseling et al., 2015). High-throughput molecular studies (Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016) identified the co-deletion of chromosomes 1p/9q arms and mutation in TERT and FUBP1 genes as genetic markers of oligodendrogliomas.

OD patients have a better prognosis than astrocytoma patients, and their mean OS ranges between 12-14 years. The tumor can remain silent for many years. However, in a selected number of cases, the development of tumors with clinical and histological
features of high malignancies (either grade III or even grade IV), occurs (Louis et al., 2016).

**Glioblastomas**

Glioblastomas (GBM) are extremely aggressive, highly malignant, and the most frequent of gliomas. Their main features include high mitotic and vascular proliferation rates, necrosis and resistance to both chemotherapy and radiotherapy treatments (Cloughesy et al., 2014). GBMs occur mainly in adults and can originate *de novo* (primary), without any previous history of a lower grade tumor, or through the malignant transformation of pre-existing tumors, which occurs in around 5% of the cases (Ohgaki and Kleihues, 2013).

Recent advances in integrated large scale strategies allowed the identification of genetic alterations singular to GBM’s genesis and progression (Brennan et al., 2013; Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016; Phillips et al., 2006; Stieber et al., 2014; Verhaak et al., 2010) (Figure 2).

![Figure 2: Main molecular alterations and signaling pathways in glioblastomas. Adapted from (Brennan et al., 2013).](image)

These genetic studies have identified four molecular subtypes of GBM: Proneural, Classical, Neural and Mesenchymal. The proneural subtype is characterized...
by alterations in \textit{IDH1/2}, \textit{TP53} and \textit{PDGFRA} genes. The prognosis for patients with this type of GBM is better, since it has been previously shown that alterations in \textit{IDH1/2} genes are an independent better prognosis factor (Yan et al., 2009). There are no specific genetic alterations detected in the neural subtype. This subtype is mainly defined by the overexpression of normal neural markers, such as GABRA1 and SYT1, as well as MBP and SNCG (Verhaak et al., 2010). The other two subtypes, Classical and Mesenchyal, present distinct molecular features, albeit similar clinical outcomes and prognosis – and both have a worse prognosis than Proneural GMB. The identity of the Classical subtype is defined by amplifications in chromosome 7 and deletions in chromosome 10, corresponding to \textit{EGFR} mutations/amplifications, such as the oncogenic variant \textit{EGFRVIII} (Thorne et al., 2016), and loss of the In\textit{k4a}/\textit{ARF} locus, respectively. The deletion of \textit{CDKN2A} gene happens in around 95% of Classical GBM cases (Verhaak et al., 2010). Finally, 57% of the Mesenchymal subtype carries mutations in the NF1 gene, mutations/deletion of RB1 gene in 13% of cases and overexpression of CHI3L1 and MET, genes associated to epithelial-to-mesenchymal transition and of a worse prognosis (Phillips et al., 2006; Verhaak et al., 2010).

Despite being prevalent in a specific subgroup, we can observe in Figure 3 that the molecular alterations associated to each GBM subtype are not exclusive. It is also worthwhile to remember that very few of these alterations happen in \textit{hotspots} – regions with an accumulation of mutations (Rogozin and Pavlov, 2003) – as is the case of \textit{IDH1} (R132H mutation) and the oncogenic variant \textit{EGFRVIII}. Therefore, to achieve precise molecular classification of GBMs one would need a global analysis of its genetic alterations.
Despite further elucidating the biology of GBMs and their complex intrinsic heterogeneity, these findings also hinder treatment options, given the plethora of possible therapeutic targets.

**Glioma microenvironment**

Glioma tumorigenicity is not exclusively the result of its genetic alterations. The crosstalk between tumor cells and the surrounding microenvironment plays a crucial role in modulating glioma growth and aggressiveness. Cells that constitute this microenvironment include cancer stem cells, endothelial cells, pericytes and normal CNS cells, such as glial cells, neurons and microglia (Charles et al., 2011). The distribution of such cells leads to the formation of specific niches, rendering the glioma microenvironment vastly heterogeneous. The consequence is the maintenance of tumor growth and resistance to immune system attacks and to treatment (Hambardzumyan and Bergers, 2015). The most abundant, non-neoplastic cells in this microenvironment belong to the myeloid lineage, comprising of CNS-resident microglia, and infiltrating tumor associated monocytes/macrophages (further called iTAMs) originating in the bone marrow (Hambardzumyan et al., 2015). A recent study showed that myeloid immune cells are the first to respond during the early phases of gliomagenesis (Chen et al., 2015).

**Microglia, origin and development**

Microglia the resident immune cells of the CNS with a unique ontogeny and are crucially shaped by their local CNS environment (Salter and Beggs, 2014). They represent around 10% of all brain cells (Ransohoff and El Khoury, 2016). Microglia were first described by the work of Rio Ortega, early in the 20\textsuperscript{th} century (Tremblay et al., 2015), where he detailed microglia cell morphology: a small cell body highly branched ramifications. During development, microglia are implicated in neuronal network formation by synaptic pruning. This monitoring function continues into adulthood and
is essential for the maintenance of homeostasis (Davalos et al., 2005; Nimmerjahn et al., 2005).

Fate mapping studies showed that microglia have a different ontogeny than other tissue macrophages (Hoefeli et al., 2015). Microglia originate from erythro-myeloid progenitors in the yolk sac and exert an assortment of functions, like antigen-presentation, phagocytosis, neural support and are implicated in shaping neural networks, further described on the next topic (Hoefeli et al., 2015; Kierdorf et al., 2013; Schulz et al., 2012). Genome-wide transcriptome and epigenome studies of mouse microglia showed that microglia cluster very differently from other tissue macrophages and other glial cells (Butovsky et al., 2014; Chiu et al., 2013; Hickman et al., 2013; Zhang et al., 2014). In mice, microglia develops from one progenitor cell, an erythro-myeloid progenitor from the yolk sac, and migrate to the brain very early in development, contrary to what happens with other tissue resident macrophages, that develop later and go through to the fetal liver before migrate to their specific tissues (Ginhoux and Guilliams, 2016; Ginhoux and Prinz, 2015; Kierdorf et al., 2013; Schulz et al., 2012). These studies also revealed that microglia genesis is dependent on the signaling of colony stimulating factor 1 receptor (CSF1R) and transcription factors PU.1 and IRF8 (Kierdorf et al., 2013).

After infiltrating the brain, these cells develop, become self-sufficient and, in homeostatic conditions, there is no infiltration from circulating monocytes/macrophages (Perdiguero and Geissmann, 2016; Ransohoff, 2011). Recent study (Elmore et al., 2014), in which there was depletion of microglial cells from the mouse brain using CSF1R inhibitors, this population was replaced by new microglia within a week after inhibition was stopped. This study revealed the existence of a Nestin-positive microglia population. Another study (Bruttger et al., 2015), using genetic ablation of microglia, showed that these cells form clusters of proliferative cells that express high levels of IL1R1, and that these cells are responsible for the repopulation of the mouse brain.

**Microglia function**

The role of microglia in monitoring the brain during homeostasis is well established, along with its function in the defense of CNS in the advent of bacterial and viral infections, lesions, neurodegenerative and auto-immune diseases (Shemer et al., 2015). During homeostasis, microglia have a small cell body and extended ramifications
in order to monitor the surrounding environment and to connect itself to synaptic and extra-synaptic regions (Tremblay et al., 2010). When activated by stimuli, whether they are physiological or pathological, microglia retracts their ramifications and become amoeboid. This process is quite fast and dynamic, and is reversible at any stage (Colton et al., 2000; Karperien et al., 2013).

Microglia express receptors on their membrane capable of measuring synaptic activity, underlying a role for these cells in the removal of “weak” synapsis (synaptic pruning) (Kettenmann et al., 2011; Paolicelli et al., 2011; Schafer et al., 2012). At the same time, other studies suggested the participation of microglia in processes such as the formations of synapses, neuronal survival and axonal growth (Parkhurst et al., 2013; Ueno et al., 2013; Wu et al., 2015).

As CNS phagocytes, microglial cells are responsible for the identification and removal of apoptotic cells, as well as acting as the first line of defense against pathogens (Ransohoff and El Khoury, 2016). Through the fractalkine receptor (CX3CR1), they are able to respond to phagocytosis stimuli sent from cells entering apoptosis, in addition to controlling the number of neurons, even being able to induce programmed cell death to those cells (Brown and Neher, 2014; Sokolowski et al., 2014). All of these mechanisms maintain the homeostatic state in the brain and confer to microglia the title of “guardian of the CNS” (Figure 4).

**Figure 4:** Microglia function as “guardians of the CNS”.

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**Tissue repair**

**Phagocytosis (clearance)**

**Antigen presentation**

**Fast response to stimuli/injury**

**Constant surveillance of its surroundings**

**Synaptic “pruning”**
Microglia under pathological conditions

The role of microglia in neurodegenerative diseases, such as Alzheimer’s Disease (Heppner et al., 2015) and Parkinson’s Disease (Sanchez-Guajardo et al., 2015), as well as in auto-immune diseases, such as multiple sclerosis (Mahad et al., 2015), has been thoroughly studied. Upon the onset of glioma, microglia respond to its stimuli as it does to any other injury. However, this interaction has raised controversy, as there is no consensus whether microglia responses to glioma are always the same, beneficial to tumor growth, or what are their variations. The focus of this work is how microglia changes in the advent of glioma, and we will present current literature on the matter in the next sections.

Infiltrated monocytes and macrophages

While the only myeloid cell in the normal brain is microglia, under neuro-pathological conditions, blood brain barrier integrity is affected and allows infiltration of myeloid cells from the periphery. Monocytes that infiltrate the brain originate from hematopoietic stem-cells in the bone marrow (Shi and Pamer, 2011) and are, therefore, ontogenetically different from microglia. However, to differentiate between these two cell types in the diseased brain has always been arduous, since there is a lack of specific markers and previous reports suggested that circulating monocytes can remain in the CNS after inflammation is resolved, and acquire microglia-like features further hampering distinction between resident microglia and infiltrated myeloid cells (Flügel et al., 2001; Hickey and Kimura, 1988; Massengale et al., 2005). This last hypothesis was contradicted by murine parabiosis experiments, where it was demonstrated that infiltrated cells disappear from the CNS when the disease enters remission (Ajami et al., 2011). For mice, it is possible to identify infiltrated monocytes using the LY-6C marker. For human, so far there is no reliable marker known to make the same differentiation.

Activation of microglia and iTAMs and their role in glioma

As mentioned before, innate immune cells such as microglia and iTAMs are major components of the glioma microenvironment, constituting to up to 40% of the tumor mass. There are conflicting studies regarding the role of such cells in tumor progression. While some claim better outcomes for patients with high rates of immune
cells, either by infiltration or from the resident tissue, many others have assessed the same phenomena to be related to poorer prognosis (reviewed by Fridman et al. (Fridman et al., 2012)). Such divergence in results seems to arise from the different functional and activation states innate immune cells can adopt within the same tumor and at different time points. Considering their ability to respond readily to stimuli, changes the microenvironment can lead both to anti or pro-tumoral responses.

Historically, microglia and iTAMs activation has been classified as classic (M1) and alternative (M2) (Galdiero et al., 2013; Hao et al., 2012; Mantovani et al., 2002). Classical activation corresponds to a pro-inflammatory phenotype, and its features include the production of iNOS, free radicals and inflammatory cytokines such as TNF-alpha and IL1-beta. The anti-inflammatory phenotype (M2, alternative) is particular to tissue repair, in which there is an increased production of arginase 1, of cell surface receptor CD163, and the release of growth factors such as TGF-beta and the hepatocyte growth factor. Gamma interferon and microbial products induce classic activation, while interleukin 4 or even TGF-beta lead to a M2 phenotype. Due to the fast way both microglia and iTAMs respond to stimuli, there is high controversy regarding the M1/M2 polarization. However, this classification still is broadly used and corresponds to clinical alterations (Ellert-Miklaszewska et al., 2013; Gabrusiewicz et al., 2015; Ghoochani et al., 2016; Nakagawa and Chiba, 2014).

Studies have demonstrated that iTAMs promote tumor growth using mechanisms that are solely immunological, but also non-immunological mechanisms; and that in the majority of cases, they present an anti-inflammatory phenotype (Mantovani et al., 2008; Pollard, 2009; Solinas et al., 2009). The same is true for microglia (Glass and Synowitz, 2014). The secreted factors in this activation phenotype promote, for instance, increased tumor angiogenesis (Brandenburg et al., 2016; Muramatsu et al., 2010), increased glioma cell motility and invasion (Bettinger et al., 2002), as well as interactions with cancer stem-cells, enhancing tumor proliferative capacity and resistance to therapy (Sarkar et al., 2014; Ye et al., 2012; Zhou et al., 2015).

Immune evasion – characterized by the ability tumor cells have to manipulate the immune system via secretion of cytokines and growth factors, and one of the hallmarks of cancer (Hanahan, 2014) to promote tumor progression and escape destruction –, is crucially dependent on the crosstalk between these two types of cells. In the scope of glioma, with such high heterogeneity rates found in tumor cells, targeting non-neoplastic cells, particularly microglia and iTAMs, seems to be a more
effective gambit to understand and overcome the mechanisms associated with recurrence and therapeutic resistance.

Despite all the evidence just cited, the great majority of the studies was done in mice, where it is possible to differentiate between microglia and iTAMs. Another aspect that hampers the extrapolation of murine results to humans is the fact that mice gliomas are not very similar to human gliomas regarding their cell of origin and molecular alterations (Szatmári et al., 2006). Different molecular subtypes of GBM have varied clinical behavior (Li et al., 2015; Natesh et al., 2015; Steed et al., 2016), and might interact differently with the microenvironment. Besides, the evaluation of expression of microglia activation markers in human gliomas needs a control population for comparison. In most cases, samples from epilepsy surgeries are used in human studies. Microglia associated to epilepsy displays an intrinsic inflammatory status that differs from normal, homeostatic microglia (Devinsky et al., 2013; Eyo et al., 2016). The use of post-mortem brain tissue is a useful alternative, provided the protocols and the characterization of the material is through.

**Aims of this thesis**

The main goal of this thesis is to study the status and phenotype of microglia in human gliomas and to delineate the human microglia gene expression profile. Microglia are essential for the homeostasis and protection of the CNS. Because of their plasticity, microglia readily respond to stimuli and become activated, exerting their functional roles. Gliomas, the most common of brain tumors, possess high percentages of those cells, along with iTAMs. The intrinsic heterogeneity of gliomas results in different responses to the microenvironment, depending on the type of tumor. Advances in large scale genetic studies have enabled the identification and characterization of different molecular subtypes of gliomas. However, there is little progress in treatment options so far. Understanding the dynamics between tumor and myeloid cells and the correlation between oncogenic molecular alterations in the tumor and the changes leading to pro-tumorigenic activation of innate immunity cells would elucidate potential treatment alternatives.

**Outline of thesis**
In this Chapter 1, we reviewed the current literature on glioma biology, classification and molecular subtyping. We also explored the knowledge in the function of cells belonging to glioma microenvironment innate immune compartment, namely microglia and iTAMs.

In Chapter 2, we analyzed the expression and correlation of genes associated with stemness and glioma stem cells (ID4, SOX4 and OCT-4), an association that imparts shorter overall survival in primary GBM patients.

In Chapter 3, we applied NGS technology to classify a Brazilian cohort of GBM samples. We assessed the correlation of our molecular findings using a more feasible proteomic immunohistochemistry-based approach. Our results indicate the need for a genetic approach to further classify GBMs, particularly the Mesenchymal subtype.

In Chapter 4, we explored the role of a family of transcription factors, inhibitors of differentiation (IDs) in gliomas from different origins (astrocytic and oligodendrocytic) and grades (I-IV), as well as the different GBM subtypes classified in Chapter 3. We show an association between IDs and the proneural subtype of GBM, as well as their usefulness in differentiating between astrocytomas and oligodendrogliomas.

In Chapter 5, we describe a protocol for ex vivo isolation of pure populations of microglia and myeloid infiltrates from the CNS, based on mechanical dissociation followed by FACS-sorting.

In Chapter 6, we identified the human microglia transcriptome and assessed how the aging process affects these cells. Aside from stipulating a core of genes responsible for human microglia identity, we also demonstrated that genes related to actin modulation are affected during aging, possibly hampering cell motility.

Finally, in Chapter 7, we report the differences found in the transcriptome of glioma and normal human microglia, as well as the differences between microglia derived from lower grade gliomas and glioblastomas. We propose a transcriptional network of regulators responsible for the proliferative and motility changes we found, as well as related the extracellular matrix genes overexpression to the most malignant subtypes of GBMs (Mesenchymal).

In Chapter 8, a summary and discussion of the principal findings from this thesis are presented, as well as an overview of possible future direction on experiments involving the cells that play an important role in glioma progression.
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factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron 82, 380–397.


