Marina Alexandra Moreira do Couto

ROLE OF MICROGLIA-MEDIATED NEUROINFLAMMATION IN DYSFUNCTION OF BLOOD-BRAIN BARRIER IN GLIOBLASTOMA

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UNIVERSIDADE DE COIMBRA

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List of Abbreviations

AJ	Adherens junctions
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
EBM-2	Endothelial Basal Medium-2
EC	Endothelial cells
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiform
GIMs	Glioblastoma-infiltrating myeloid cells
GLUT-1	Glucose transporter-1
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gp130	Glycoprotein 130
HRP	Horseradish peroxidase
IDH	Isocitrate dehydrogenase
IFN-ɣ	Interferon-ɣ
IL	Interleukin
IL-6R	Interleukin-6 Receptor
iNOS	inducible nitric oxide synthase
JAM	Junctional adhesion molecules
JAK	Janus kinases
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor

MG	Microglia
MG/MP	Microglia and Macrophages
MHC	Major histocompatibility complex
MIF	Macrophage inhibitory factor
MMP	Matrix metalloprotease
NF-$_K$B	Nuclear Factor-$_K$B
NF1	Neurofibromatosis type 1
NO	Nitric oxide
PFA	Paraformaldehyde
PI-3	Phosphatidylinositol-tri-phosphate kinase
PGE2	Prostaglandin E2
Pgp	P-glycoprotein
p-STAT	Phosphorylated STAT3
PTEN	Phosphatase and tensin homolog
sIL-6R	Soluble form of IL-6Rα
RITC	Rhodamine B isothiocyanate
ROS	Reactive oxygen species
RT	Room temperature
STAT	Signal transducer and activator of transcription
TEER	Transendothelial Electrical Resistance
TGF-β	Transforming growth factor- β
TJ	Tight junctions
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor- α
TP53	Tumor protein p53
VEGF	Vascular endothelial growth factor
VE-cadherin	Vascular endothelial cadherin
WHO	World Health Organization
ZO	Zonula occludens

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Resumo

A microglia (MG) tem um papel importante na neuroinflamação protegendo o sistema nervoso central (CNS, do inglês *cental nervous system*) contra agentes patogénicos e lesões. No entanto, num microambiente tumoral cerebral, a MG adquire um fenótipo de ativação M2 distinto que promove o crescimento e invasão tumoral, através da libertação de citocinas e outros mediadores inflamatórios. Recentemente, a ativação da MG e consequente neuroinflamação parece estar relacionada com a disfunção da barreira hematoencefálica (BBB, do inglês *blood-brain barrier*) geralmente observada em várias doenças do CNS, incluindo tumores cerebrais. No entanto o papel da resposta inflamatória desencadeada pela interação da MG com as células tumorais na disfunção da BBB não é conhecido.

Este trabalho teve como principal objetivo avaliar o efeito das interações reciprocas entre a MG e as células tumorais de glioblastoma (GBM) na integridade da monocamada de células endoteliais (EC, do inglês *endothelial cells*).

A exposição da monocamada de células humanas endoteliais hCMEC/D3 ao meio condicionado recolhido da co-cultura de células da MG BV2 com células de GBM U87, induziu uma diminuição da resistência eléctrica transendotelial (TEER, do inglês *transendothelial eletrical resistance*) e um aumento na permeabilidade da monocamada a dextranos de 4 kDa e 70 kDa, em relação ao controlo. Estes efeitos foram acompanhados pela diminuição e alteração na distribuição celular da claudina-5 e da ZO-1, elementos importantes na estrutura das *tight junctions*. Além disso, a interação dinâmica entre a MG e as células tumorais desencadeou a libertação de IL-6 e consequente activação da via JAK/STAT. O bloqueio da actividade da IL-6 através da inibição da via JAK/STAT com AG490, preveniu a disfunção das EC.

Em conclusão, os nossos resultados sugerem que a IL-6 presente no meio condicionado da co-cultura de MG-GBM afeta a integridade da monocamada de EC.

Em geral, demonstramos que a MG, sob a influência de células de GBM, induz alterações na integridade da monocamada de EC com ruptura das *tight junctions*,

através da libertação de IL-6 e consequente activação da via de JAK/STAT. Estes resultados fornecem novos dados sobre os mecanismos subjacentes às alterações na permeabilidade da BBB no GBM.

Palavras-chave: glioblastoma, microglia, barreira hematoencefálica, células endoteliais, interleucina-6.

Abstract

Microglia (MG) has an important role in neuroinflammation protecting the central nervous system (CNS) against pathogens and injuries. However, in a brain tumor microenvironment, MG acquire a distinct M2 activation phenotype that promote the tumor growth and invasiveness through the release of cytokines and other inflammatory mediators. Recently, microglial activation and consequent neuroinflammation might be related with the blood-brain barrier (BBB) dysfunction commonly observed in several CNS diseases, including brain tumors. Several studies pointing out for the role of activated MG in BBB disruption, however the role of the inflammatory response triggered by the interaction between MG and glioma cells in BBB dysfunctions is unknown.

In this work we evaluated the effects of reciprocal interactions between MG and GBM cells in the integrity of endothelial cells (EC) monolayer.

The exposure of human endothelial hCMEC/D3 cells monolayer to conditioned medium harvested from the co-culture of BV-2 microglia with U87 glioblastoma induced a decrease in the transendothelial electric resistance (TEER) and an increase in 4 kDa and 70 kDa-dextrans permeability across the EC monolayer in relation to control cells. These effects were accompanied by a decrease in the expression and changes in the cellular distribution of claudin-5 and zonula occludens (ZO) -1 that are important elements to tight junction structure. Moreover, the dynamic interaction between MG and tumor cells triggered the release of IL-6 and consequent activation of JAK/STAT. The blockade of IL-6 activity via inhibition of JAK/STAT pathway with AG490, prevented the EC dysfunction.

Overall, we demonstrated that MG under the influence of GBM cells, impaired the integrity of the EC monolayer by disrupting of tight junctions, through the release of IL-6 and subsequent activation of the JAK/STAT pathway. These results provide new insights into the mechanisms underlying the disruption of BBB permeability in GBM.

Keywords: glioblastoma, microglia, blood-brain barrier, endothelial cells, interleukin 6.

Introduction

1. Introduction

1.1. Glioma

Glioma is the most common and lethal brain tumor corresponding to more than 70% of all tumors of the central nervous system (CNS) (Louis *et al.,* 2007; Ohgaki & Kleihues 2009). This tumor originates in the glial cells that are non-neuronal cells with several functions, such as maintain homeostasis, supply nutrients to neurons, protect the CNS, and are also involved in complex processes like signal transduction and neurotransmission (Meir *et al.,* 2010).

According to the World Health Organization (WHO), gliomas can be classified depending on the cellular origin and morphological features, into astrocytomas, oligodendrogliomas, ependymomas and oligoastrocytomas (mixed gliomas) (Louis *et al.,* 2007). Histopathological classification of gliomas is based on morphological similarities of tumor cells with normal brain cells. Tumors with astrocytic features that arise from astrocytes are classified as astrocytomas; while tumors with oligodendroglial features, that arise from oligodendrocytes or from a glial precursor cell, are classified as oligodendrogliomas. When gliomas present morphological features of both astrocytes and oligodendrocytes are classified as oligoastrocytomas. The tumors with similar characteristics to ependymal cells are classified as ependymomas (Meir *et al.,* 2010).

In a malignancy scale, depending on histopathological features, nuclear morphology, mitotic activity, necrosis, and vascular proliferation, gliomas are classified into different grades: oligodendrogliomas and oligoastrocytomas are classified as grade II and grade III; astrocytomas, that are subdivided as pilocytic, diffuse and anaplastic, are classified as grade I, II and III, respectively; and glioblastoma multiforme are classified as grade IV. A higher grade corresponds to an increased malignancy. The tumor grade can influence the choice of therapies, determining the use of adjuvant radiation and specific chemotherapy protocols (Jovčevska *et al.,* 2013; Louis *et al.,* 2007).

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Glioma have an annual incidence of 5 cases for 100 000 individuals. These tumors are more common in men than women, occurring more frequently during the adult phase, especially among the elderly. Glioma occurrence are higher in non-Hispanic people than in American Indian/Alaskan natives. Looking to the globe, countries like Australia, Canada, Denmark, Finland, Sweden, New Zealand and United States of America report a high incidence of gliomas, while countries like Philippines and India report lower incidence of the disease (Schwartzbaum *et al.,* 2006).

The most common and aggressive glioma is glioblastoma multiforme (GBM) with a percentage rate of all gliomas of 60-70%. In Europe and North America, the annual incidence of GBM is 2-3 new cases per 100 000 individuals. Anaplastic astrocytomas are 10-15% of all gliomas, anaplastic oligodendrogliomas and anaplastic oligoastrocytomas are 10%, and the less common tumor types, such as anaplastic ependymomas and anaplastic gangliogliomas, are the remaining 5-20% of all gliomas (Jovčevska *et al.,* 2013).

Glioma risk factors have been associated with advancing age, male sex, caucasian race and exposure to high-dose radiation. Some studies have also associated an increased glioma risk with severe head injury, dietary risk factors with N-nitroso compounds, alcohol and tobacco. Preliminary evidences have shown that adults with a history of allergies (e.g. asthma) and highly levels of serum IgE have a decreased incidence of glioma. Genetic predisposition has been also indicated as a glioma risk factor in 5-10% of glioma cases, due the presence of inherited mutations of genes associated with rare syndromes (e.g., neurofibromatosis 1 and 2, tuberous sclerosis, retinoblastoma, Li-Fraumeni syndrome, Turcot syndrome and multiple hamartoma syndrome) (Schwartzbaum *et al.,* 2006).

Glioma patients present symptoms, that depend on the anatomical site of the glioma in the brain, such as headaches; nausea or vomiting; changes in speech, vision, hearing, or balance; mood and personality alterations; convulsions; and memory deficits (Gladson *et al.,* 2010).

1.2. Glioblastoma multiforme

GBM is the most common and deadliest brain tumor, and represent about 60-70% of all gliomas. This tumor is named glioblastoma multiforme due the pleomorphic variation in size and shape of the tumor cells. GBM patients have a poor prognosis with a median survival of 12-15 months, and when GBM is recurrent the median patient survival reduces to 3-6 months, having a worse prognosis. GBM occurrence is more common in men than in woman, and the majority of patients are aged in the range of 55-87 years (Jovčevska *et al.,* 2013).

According to the WHO, GBM is classified as astrocytoma, because originates in the glial cells, namely in astrocytes, and is a grade IV tumor in the malignancy scale (Haiyan 2011; Ohgaki and Kleihues 2009). This tumor is characterized by genetic complexity, increased mitotic activity, vascular proliferation, areas of necrosis and high invasive ability. Despite GBM do not perform metastasis, tumor cells have the ability to infiltrate into adjacent healthy brain areas which difficult the treatment (Gladson *et al.,* 2010; Meir *et al.,* 2010).

GBM develops into 2 different ways: primary (or *de novo*), that develops without clinical or histological evidence of any precursor neoplastic lesion; and secondary, that develops from low-grade (II or III) diffuse astrocytoma or anaplastic astrocytoma. The primary GBM corresponds to more than 90% of the cases, develops very rapidly and affects older patients with a mean age of the 62 years. The secondary GBM corresponds to less than 10% of the cases, develops more slowly and affects younger patients with a mean age of the 45 years (Dimov *et al.,* 2011; Gladson *et al.,* 2010; Ohgaki and Kleihues 2009). The primary GBM is more common than secondary GBM, and despite a similar histologic appearance, both GBM are distinct tumors entities that differ in their genetic and epigenetic profiles, have different response to therapy and different prognosis (Jovčevska *et al.,* 2013; Ohgaki & Kleihues 2013).

Recently GBM were classified into gene expression-based subtypes. These include classical, mesenchymal, neural and proneural subtypes. The classical GBM subtype has a characteristic profile of highly proliferative cells, characterized by

chromosome 7 gain and chromosome 10 loss, that leads to amplifications or mutations of the epidermal growth factor receptor (EGFR), and loss or mutation in the phosphatase and tensin homolog (PTEN) tumor suppressor gene. The mesenchymal GBM subtype, associated with a high angiogenesis, is characterized by loss or mutation of neurofibromatosis type 1 (NF1) gene, tumor protein p53 (TP53), PTEN genes and high expression of genes of tumor necrosis factor (TNF) family and nuclear factor-kB (NF-kB) pathway. The proneural GBM subtype is characterized by frequent mutations in isocitrate dehydrogenase (IDH) genes 1 and 2, and in TP53. The neural GBM subtype express proteins associated with neuronal differentiation, and shows features intermediate between proneural and mesenchymal tumors. Mutations in IDH1/2, associated with hypermethylation phenotype, have been used as a genetic marker for secondary GBM, due the high frequency of this mutation in secondary GBM (>80%) and low frequency in primary GBM (<5%) (Jovčevska *et al.,* 2013; Ohgaki & Kleihues 2013; Schwartzbaum *et al.,* 2006).

GBM are composed of a heterogeneous mix of neoplastic and non-neoplastic cells, such as fibroblasts, endothelial and immune cells, that can be native and recruited cells (Dimov *et al.,* 2011). This tumor can be highly infiltrated by cells of myeloid origin, namely microglia (MG) and macrophages, which are referred as glioblastoma-infiltrating myeloid cells (GIMs). GIMs can represent up to about 30% of the tumor mass and there is a positive correlation between MG infiltration in GBM and the malignancy of the tumor. Recently, have been shown that GIMs play several roles in GBM progression including proliferation, survival, motility and immunosuppression (Coniglio & Segall 2013).

1.2.1. Therapeutic approaches

The first step in the treatment of GBM tumor is the surgical resection that can be total or partial, since the neurosurgeon only removes the tumor mass of sites that are accessible, thereby preventing damage in normal brain tissue. The surgical resection improves the survival and life quality of the patient and allows get tumor tissue for diagnosis and choice of treatment. To improve the success of treatment rate, due to the high probability of local recurrence, the surgical resection is combined with

radiotherapy and chemotherapy (Holland 2000). Currently, there are additional treatment options, still experimental procedures, such as passive and active immunotherapy, use of angiogenesis inhibitors in combination with chemotherapeutics and gene/antibody therapy (Jovčevska *et al.,* 2013). However these additional treatment options have been unsuccessful in curing this disease due the presence of blood-brain barrier (BBB) that prevents the passage of molecules >500 Da from entering the brain (Dubois *et al.,* 2014; Jovčevska *et al.,* 2013). To pass through the BBB, the chemotherapy drugs used in GBM treatment must have the following characteristics: low molecular weight; high lipid solubility; low ionization and minimal protein binding capability (Zang *et al.,* 2015). Temozolomide is the most common chemotherapy drug used in GBM treatment, in combination with surgery and/or radiotherapy, since this small alkylating agent form O^6-methylguanine in DNA which miss-pairs with thymine during the next DNA replication cycle, leading to the cancer cells death (Okada *et al.,* 2009).

Even with an aggressive treatment, the median survival rate of GBM patients is 12-15 months, that depend on the patient's age when appeared the first symptoms, the patient's conditions at the time of surgery, the tumor resection performed (total or partial), and the radiation dose used in the treatment (Furnari *et al.,* 2007; Meir *et al.,* 2010; Sousa *et al.,* 2002; Zhai *et al.,* 2011).

1.3. Microglia

The innate and adaptive immune systems recognize and protect tissues from infections and damage, and have been implicated in promoting and preventing tumor growth. MG, a distinct class of glial cells, is known to play an important role in supporting and sustaining neuronal functions (Streit 2002; Zlokovic 2008). These cells are derived from a monocyte/macrophage lineage and are referred to as the macrophages of the CNS, being the first line of defense against a variety of stressors (Okada *et al.,* 2009; Tremblay *et al.,* 2011). MG play a critical role in the innate and adaptive immune responses of CNS, initiating appropriated responses (Zlokovic 2008).

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In a normal condition, MG can constitute up to 10% of cells in the CNS, while in a tumor brain, the infiltrated MG can represent approximately 30% of the total tumor mass. In adult brain, MG can present two states: resting surveillance and activated. Resting surveillance MG presents a ramified form with small bodies and long and thin processes. These ramified branches allow the communication with surrounding neurons and other glial cells. When activated, the ramified form transform into an amoeboid form that responds to infectious and traumatic stimuli though the production of a variety of inflammatory mediators [such as cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO)] which contribute to de clearance of pathogenic infections (Kettenmann *et al.,* 2011; Zlokovic 2008).

The activation states of MG are classically divided into two distinct phenotypic profiles: M1 or classical activation and M2 or alternative activation, which differ in gene expression profiles, activating signals, cytokine/chemokine production, receptor expression, and biological effects (Figure 1) (Kofler & Wiley 2011; Li & Graeber 2012).

The classic activated M1 phenotype is characterized by the expression of the signal transducer and activator of transcription (STAT) 1 and the production of inducible nitric oxide synthase (iNOS). This phenotype is capable of stimulating anti-tumor immune responses by producing elevated expression of pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β, IL-18, IL-12; major histocompatibility complex (MHC) class II and co-stimulatory molecules CD80 and CD86 (Kofler & Wiley 2011; Li & Graeber 2012). M1 phenotype have enhanced endocytic functions and enhanced ability to kill intracellular pathogens, however, although potentiate the tumor suppression, this phenotype can also cause damage to healthy tissue as a side effect. Once activated, it is important that the inflammatory process and macrophages be turned off, which is maintained by the ubiquitination of cell surface toll-like receptors (TLRs), production of suppressor cytokines, production of micro-ribonucleic acids to decrease cellular transcription, up-regulation of receptor antagonists, and release of receptors for cytokines to decrease their overall signaling (Martinez *et al.,* 2008).

The alternatively activated M2 phenotype is characterized by the expression of STAT3 and surface CD163 and CD204, and by the production of arginase, used as a molecular marker to this phenotype. M2 phenotype promote tissue repair and remodeling, angiogenesis, tumor promotion, and act to suppress tissue-destructive immune reactions. M2 activated MG typically secrete anti-inflammatory cytokines (such as IL-4 and IL-10), transforming growth factor-β (TGF-β), membrane matrix metalloproteases (MMP) type 1 and 12, and low levels of NO production (Kofler & Wiley 2011; Gabrusiewicz *et al.,* 2011). This alternative activation was recently subdivided in different categories with associated functional differences. These different categories acquire several nomenclatures because this field is still evolving, what makes difficult the interpretation and comparison of the findings. M2 phenotype can be subdivided in **M2a** (or alternatively activated macrophages) elicited by type II cytokines IL-4 or IL-13; **M2b** (or type II activated macrophages) obtained by combination with IL-1β or lipopolysaccharide (LPS); and **M2c** which includes deactivation programs elicited by IL-10 or TGF-β (Martinez *et al.,* 2008).
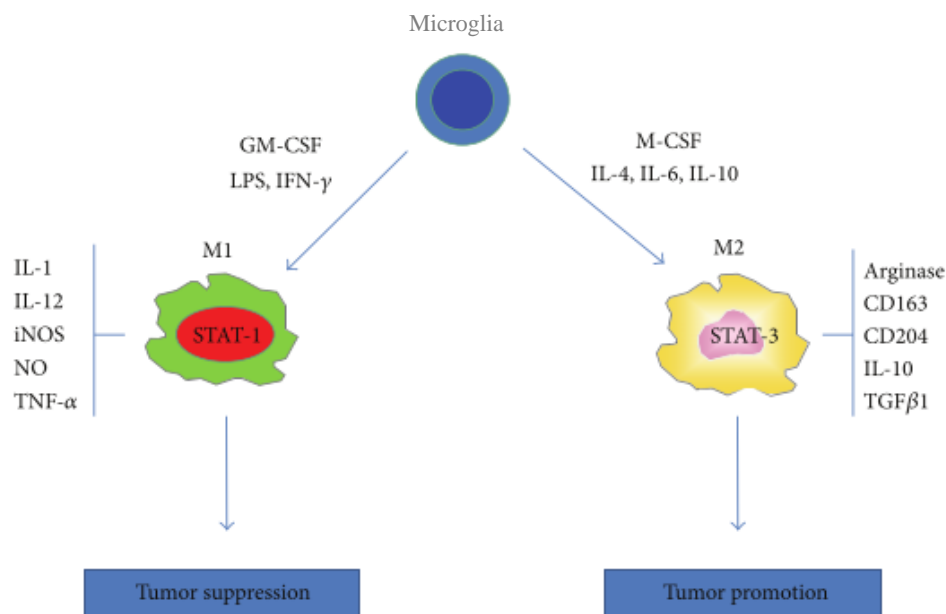
Figure 1 - Activated microglia present two distinct phenotypes when exposed to certain stimulating factors. In the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), lipopolysaccharide (LPS) and interferon-ɣ (IFN-ɣ), microglia present the M1 phenotype that produce pro-inflammatory factors leading to tumor suppression. In the presence of macrophage colony stimulating factor (M-CSF), interleukin (IL)-4, IL-6, and IL-10, microglia present the M2 phenotype that produce anti-inflammatory factors leading to tumor promotion. (Adapted from Wei *et al.,* 2013).

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Despite this separation of MG activation phenotypes, there is also evidence to suggest that there may be transitions from one phenotype to another (Kofler & Wiley 2011). The transition between activation states suggests that chronic inflammation may be a mixture of both classically and alternatively activated cells, and in an injury condition, the balance between these pathways determine the protective or deleterious role of microglial activation (Li *et al.,* 2007; Schwartz *et al.,* 2006).

In a brain tumor disease, the activation state of MG is highly influenced by the local microenvironment (Kofler & Wiley 2011). Microglial cells are able to change their phenotype according to the tumor type and stage of development (Roggendorf *et al.,* 1996). During the development of GBM, microglial cells are recruited to the tumor mass to produce cytokines and other mediators that promote the tumor growth and progression. So, MG have an important role in glioma growth and invasion (Li & Graeber 2012; Watters *et al.,* 2005).

Glioma cells are capable to produce self-supportive factors and their corresponding cell surface receptors, and promoting to their own proliferation, migration, angiogenesis and tumor extension (Kennedy *et al.,* 2013). Glioma secretes factors that suppress immune cells, such as, IL-4, IL-6 and IL-10, macrophage inhibitory factor (MIF), prostaglandin E2 (PGE2) and TGF-β, leading to the suppression of the pro-inflammatory M1 phenotype and promoting the M2 phenotype of MG that produce anti-inflammatory factors, which in turn leads to the tumor progression (Wei *et al.,* 2013). So, glioma tumor patients have the immune system in a highly suppressed state because gliomas suppress immune responses by MG, such as phagocytosis, even when MG are activated (Dimov *et al.,* 2011; Haiyan 2011). The effects of these cytokines are not only on the immune system, but also on the tumor cell. PGE2, a product of arachidonic acid metabolism, is produced at sites of inflammation or tissue damage, where it has some effects, including the enhancement of vascular permeability. PGE2 seems have also a profound modulatory effect on T-cell activation. The TGF-β is responsible for complex immunosuppressive effects, such as, inhibition of maturation and antigen presentation by dendritic cells or other antigen-presenting cells, inhibition of T-cell activation, and differentiation towards effector cells (either cytotoxic cells expressing perforin or Th1 or Th2 cells) (Okada *et al.,* 2009).

There is a great interest in therapies that modulate MG activity or function because GIMs have been proposed to promote glioma growth by secreting growth factors, immune-suppressive cytokines and angiogenic factors (Demuth *et al.,* 2007; Wagner *et al.,* 1999; Wesolowska *et al.,* 2008).

1.4. Blood-Brain Barrier

BBB is a complex and dynamic interface between the blood and the CNS. This barrier is formed by endothelial cells (EC), basement membrane, pericytes and astrocyte endfeet that together with the surrounding neurons and MG constitute the neurovascular unit that are essential for the normal function of the CNS (Figure 2) (Cardoso *et al.,* 2010).
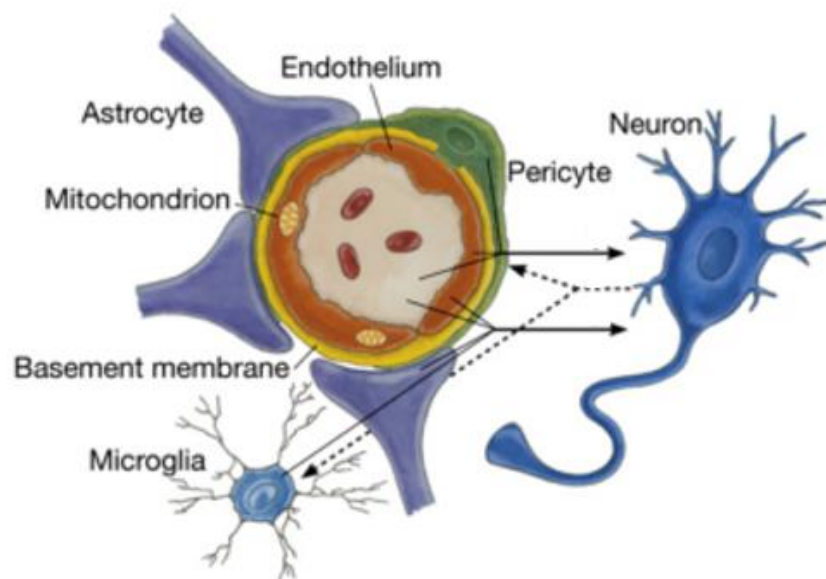
Figure 2 - Illustrative scheme of the neurovascular unit. (Adapted from Zlokovic, 2008).

The BBB has several important functions: maintains the CNS homeostasis, thought the regulation of ion balance and compounds influx/efflux (Cardoso *et al.,* 2010; Vries *et al.,* 1997); supply of nutrients by specific transport systems; protect the brain from extracellular environment by limiting the entry of toxins, pathogens, and immune cells into the neural tissue (Daneman 2012); regulate the movement of molecules, ions, and cells between the blood and the neural tissue (Daneman 2012;

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Rubin and Staddon 1999; Zlokovic 2008); and direct inflammatory cells to act in response to changes in local environment (Persidsky *et al.,* 2006).

EC play a key role in BBB properties due the presence of endothelial junctions that are transmembrane adhesion proteins which together with their intracellular partners adhere one endothelial cell to another. (Bazzoni & Dejana 2004) Brain EC differ from those of non-neuronal tissues since they are highly polarized, show absence of fenestrations, have limited movement of molecules and ions between cells (paracellular flux) due the presence of more extensive tight junctions, and also have low rates of pinocytosis/transcytosis, which limits the movement of molecules through the cell (transcellular flux) (Ballabh *et al.,* 2004; Daneman 2012).

There are three types of intercellular junctions namely tight junctions (TJ), adherens junctions (AJ) and gap junctions, that mediate the adhesion and the communication between adjacent cells. Intercellular junctions are dynamic structures since during the organization of an endothelial monolayer the contacts of cell-to-cell follow different steps of maturation (Bazzoni & Dejana 2004).

TJ (Figure 3A) have an important role on barrier properties of EC. These proteins are concentrated in the luminal side of lateral plasma membrane and act as seal that regulates lateral diffusion between the apical and basolateral plasma membrane, which limit the paracellular permeability. TJ are consisted by members of the claudin family, occludin, and junctional adhesion molecules (JAM) (Ballabh *et al.,* 2004).

Claudins are integral membrane proteins responsible for permeability restriction (Zlokovic 2008). The overexpression of claudin can induce cell aggregation and formation of TJ-like structures. These proteins can be present in cells in different isoforms (e.g. claudin -1, -3, -5, -12 and -18) that confer different size and charge selectivity qualities that result in specific barrier functions. The claudin-5 is related with the maintenance of the BBB functions and seems to have a role in angiogenesis and in diseases with increased vessel permeability (Cardoso *et al.,* 2010).

Occludin, a transmembrane TJ protein, was the first integral membrane protein discovered and is the best known. This protein has an important role in the formation

of a very close contact between adjacent cells. The presence of occludin high levels ensure decreased paracellular permeability and high resistance of the brain microvascular endothelial cells monolayers. (Persidsky *et al.,* 2006) However, occludin must be seen more in a regulatory context than as a major structural protein in the establishment of the barrier properties (Wolburg & Lippoldt 2002), since the lack of occludin does not affect the TJ because other proteins compensate well for occludin loss (such as claudin-3, zonula occludin (ZO) -1, ZO-2, vascular endothelial cadherin (VE-cadherin), and α-catenin) (Saitou *et al.,* 2000).

JAM are involved in different interendothelial junctions. The JAM more expressed in brain is JAM-1 that is involved in cell-to-cell adhesion and in organization of the tight junctional structure (Cardoso *et al.,* 2010).

ZO is located in the cytoplasmic side of the EC membrane delimiting the interendothelial cleft. This protein act as a central organizer of TJ complex allowing the connection of transmembranous TJ proteins with the actin cytoskeleton. ZO is mostly expressed in endothelial and epithelial cells that form TJ. Therefore, the epithelial cells with lack of all ZO molecules resulted in deficient TJ, which could be reversed by production of ZO-1 or ZO-2 (Daneman 2012). Loss or dissociation of ZO-1 from the junctional complexes is associated with increased barrier permeability (Cardoso *et al.,* 2010).

The AJ (Figure 3B) are located below TJ in the basal region of lateral plasma membrane. These junctions are formed by transmembrane homophilic adhesion proteins organized in multimeric complexes at the cell borders. AJ are responsible for mediate: the adhesion of EC to each other, the contact inhibition during vascular growth and remodeling, the initiation or cell polarity, and the regulation of paracellular permeability. VE-cadherin and catenin are constituents of AJ that promote the cell-cell adhesion through actin filaments linking. Cadherin is the major transmembrane component of AJ. VE-cadherin is a specific cadherin expressed by EC, that cannot be found in any other cell type. VE-cadherin mediates cell adhesion in a Ca^{2+}-dependent manner, inhibits cell proliferation, and when are overexpressed decreases cell permeability and migration. However, this protein alone is insufficient to promote

junction formation, and must to be linked to a group of proteins termed catenins (α, β and γ) through their cytoplasmatic tails, thereby allowing the link of cadherin to the actin cytoskeleton (Bazzoni & Dejana 2004; Cardoso *et al.,* 2010; Rubin & Staddon 1999).

The gap junctions are transmembrane hydrophilic channels that allow the direct exchange of ions and small molecules between adjacent cells. These junctions can establish homotypic (endothelial to endothelial) or heterotypic (endothelial-smooth muscle cells, endothelial-macrophages) communications (Dejana *et al.,* 1995).
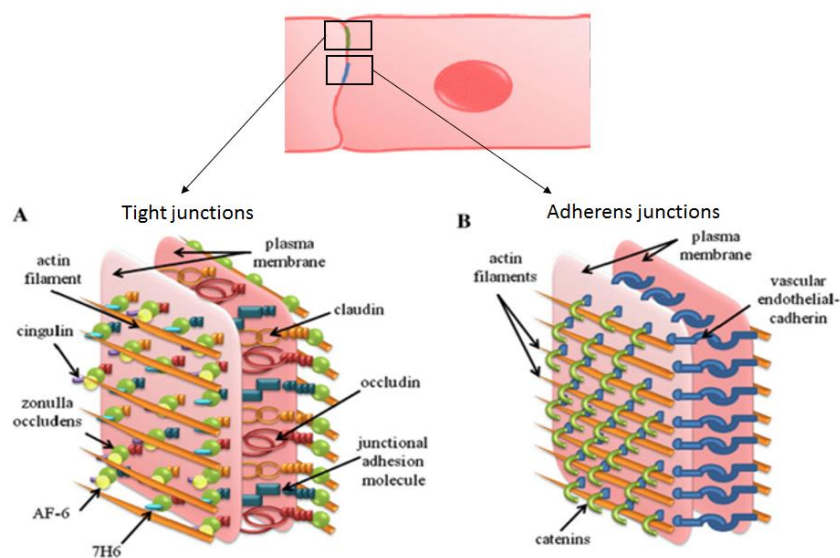
Figure 3 - Tight junctions (TJ) and adherens junctions (AJ) are proteins that allows the adhesion between endothelial cells of the central nervous system. A, TJ form a complex of parallel, interconnected, transmembrane and cytoplasmatic strands of proteins. **B**, AJ are composed of transmembrane glycoproteins linked to the cytoskeleton by cytoplasmatic proteins. (Adapted from Cardoso *et al.,* 2010).

To an optimal brain function is important maintain the BBB integrity (Vries *et al.,* 1997). The intercellular junctions are responsible for concede a low paracellular permeability and a high electrical resistance of BBB (Bazzoni & Dejana 2004). In healthy conditions, only small and lipophilic molecules can cross the BBB. However, there are some small and large hydrophilic molecules (e.g. essential nutrients, such as glucose and certain amino acids) that can enter in brain by active transport and specific transporter mechanisms (Daneman 2012; Rubin and Staddon 1999). One of these specific membrane transporter proteins is the glucose transporter-1 (GLUT-1) that allow the passage of glucose to the brain. The GLUT-1 has an asymmetrical

distribution in EC allowing a homeostatic control for glucose influx into the brain avoiding the accumulation of higher levels of glucose in brain than in the blood (Cardoso *et al.,* 2010). In a brain disease condition the intact structure of BBB is a major obstacle for the pharmacological treatment of CNS disorders, since about 98% of small molecule drugs and 100% of large molecule drugs are not able to cross the BBB and reach the CNS (Cardoso *et al.,* 2010; Zlokovic 2008).

During various pathological conditions (such as ischemic injury, intracerebral hemorrhage, trauma, neurodegenerative process, inflammation, or vascular disorder) the permeability of the BBB may be altered, leading to changes in BBB functions. The increased permeability of BBB is related to the opening of TJ, enhanced pinocytotic activity, formation of transendothelial channels and decrease in BBB-associated pericytes (Rascher *et al.,* 2002; Vries *et al.,* 1997). Due the increase of BBB permeability, plasma components and immune molecules can migrate into the brain and generate neurotoxic products that compromise synaptic and neuronal functions, leading to neuronal dysfunction (Daneman 2012; Hawkins & Davis 2005; Vries *et al.,* 1997).

At the early stage of glioma development, the BBB remains intact, limiting drug transport into the brain by serving as a physical (TJ), metabolic (enzymes) and immunological barrier. However, during the development and progression of gliomas, the integrity of BBB is compromised and spatially heterogeneous even in the presence of a single tumor tissue (Zang *et al.,* 2015). The gradual progression of the glioma causes brain edema, taking to vasodilation and BBB dysfunction, in particular to the enhanced permeability of the BBB (Okada *et al.,* 2009) due abnormal structural features (Zang *et al.,* 2015). A study done by *Stewart et al.,* in a rat glioma model, show that approximately 60% of the tumor vessel profiles have junctional abnormalities and 30% have one or more fenestrations (Stewart *et al.,* 1985). Abnormalities in structural and functional components of the vessels in the tumor probably contribute to barrier breakdown. Recent studies have shown that the BBB in patients with GBM is highly compromised affecting also normal regions of the brain (Okada *et al.,* 2009).

Introduction

Different molecules have been identified to be important for BBB breakdown in many different CNS disorders (including stroke, multiple sclerosis and brain traumas), such as vascular endothelial growth factor (VEGF), ROS, inflammatory cytokines (IL-1, IL-6, TNF-α), proteases (MMP-2, MMP-9), and leukocyte adhesion molecules (P-selectin, E-selectin, Icam1, Vcam1). VEGF is an angiogenic factor that regulates the formation of blood vessels during the development, allows the homeostatic maintenance of the vasculature (Daneman 2012) and can increase endothelial permeability (Davies 2002). Have been demonstrated that inflammatory cytokines increase BBB permeability during disease (Daneman 2012). TNF-α, an inflammatory cytokine that has been identified to be elevated in multiple sclerosis patients, caused an opening of BBB inducing gaps in EC by loss of VE-cadherin (Ozaki *et al.,* 1999). Recently, neuroinflammation has gained importance in causing disturbance of BBB, since impairments in BBB permeability has been observed in this context and substances that affect the tumor growth (e.g. VEGF) also have been affect the BBB permeability (Provenzale *et al.,* 2005).

1.5. Neuroinflammation

Inflammation is a protective physiologic response to injury and infection that remove detrimental stimuli and initiate tissue healing (Schwartz *et al.,* 2006). This process is commonly observed as a mechanism of defense triggered to protect the organism from pathogens (Hodge *et al.,* 2005). Inflammation is associated with the invasion of leucocytes into injured or infected tissues, and is also tightly associated with the activation of the immune system (Allan & Rothwell 2003). In the absence of leukocyte infiltration, the inflammation is powered by tissue-resident macrophages (Schwartz *et al.,* 2006). Despite the protective effects of inflammation, common inflammatory mechanisms may contribute to many neurodegenerative disorders (Allan & Rothwell 2003).

The inflammatory response in the CNS, namely neuroinflammation, have some particularities when compared with inflammation in other organs. MG, the primary glial cells implicated in inflammation, are rapidly activated in response to infection, inflammation and injury, and acquire the morphology of activated

macrophages, limiting the area of injury through phagocytosing dying cells, and release of pro-inflammatory cytokines, such as IL-1, IL-6, IL-18, and TNF-α (Streit 2002). Generally, inflammation is beneficial to the organism because limit the survival and proliferation of invading pathogens and promote the tissue survival, repair and recovery. However, the extensive, prolonged or unregulated inflammation is highly damaging to the brain, leading to the cell damage in neurodegenerative diseases rather than have a defensive role (Allan & Rothwell 2003; Schwartz *et al.,* 2006).

Neuroinflammation seems to play a role in BBB permeability (Abbott 2000). During an injury situation the permeability of BBB is increased, allowing the migration of leukocytes into the brain that will trigger signal transduction cascades leading to loss of TJ including occludin and ZO and consequently to BBB breakdown (Raivich *et al.,* 1999; Streit 2002).

In brain tumors, inflammation can promote tumorigenesis via increased genetic alterations, resulting from macrophage-secreted reactive oxygen and nitrogen species. During the inflammatory response, some cytokines promote tumor growth by inducing angiogenesis, or by triggering signaling cascades that activate NFκB and STAT3. In a tumor microenvironment, depending on the combination of immune cells present and the signaling factors secreted, inflammation can inhibit or promote tumor growth (Galvão & Zong 2014).

1.5.1. Cytokines

Cytokines are regulatory proteins produced by many different cell types (e.g. glial cells and neurons) which contribute to initiation, propagation and regulation of inflammatory reactions in CNS through the control of neuronal and glial activation and plasticity, and also by regulating the communication between CNS and immune system (Benveniste 1998). These proteins can be classified as proinflammatory (e.g. IL-1β, IL-6, IL-18, TNF-α) and as anti-inflammatory (e.g. IL-4, IL-10 and TGF-β) (Borish & Steinke 2003).

Cytokines bind and stimulate cellular receptors that are associated with tyrosine kinases known as Janus kinase (JAK) (Hodge *et al.,* 2005). The inflammatory

cytokine IL-6 have a crucial role in GBM development. This cytokine through the activation of STAT3 promote the invasion and migration of GBM cells (Yeung *et al.,* 2013). A study done by *India et al.,* characterize IL-6 as a messenger that enhances GBM cellular heterogeneity and tumor growth (India *et al.,* 2010).

1.5.2. Interleukin-6

IL-6 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory properties (Borish & Steinke 2003; Scheller *et al.,* 2011). It is involved in several biological responses, such as immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, survival, and differentiation (Hirano *et al.,* 2000; Hodge *et al.,* 2005).

The sources of IL-6 synthesis are mononuclear phagocytic cells, T and B lymphocytes, fibroblasts, EC, keratinocytes, hepatocytes, and bone marrow cells (Borish & Steinke 2003). This protein mediates the activation, growth and differentiation of T-cells, resulting in the promotion of tissue inflammation (Chang *et al.,* 2005). IL-6 induces the pyrexia and the secretion of acute phase proteins. On the other side, IL-6 can have a role on immunosuppression because it can inhibit the differentiation of dendritic cells allowing tumor cells to escape from recognition of the immune system (Rolhion *et al.,* 2001).

The secretion of IL-6 can be induced by IL-1β, TNF-α, PGE-2, LPS and VEGF (Hodge *et al.,* 2005).

1.5.2.1. IL-6 Signaling Pathways

The receptor for IL-6 is a heterodimeric complex made by the IL-6 receptor α (IL-6R, also called CD126 or gp80), that is a glycosylated type I membrane 80 kDa protein, and by the common signal transducing receptor glycoprotein 130 (gp130) (Benveniste 1998; Scheller *et al.,* 2011; Weissenberger *et al.,* 2004).

The classic signaling of IL-6 (Figure 4a) is initiated when IL-6 binds to its membrane-bound IL-6R forming a complex that binds to the signal transducing protein gp130, thereby inducing its dimerization and initiation of the intracellular signaling

that is propagated by JAK (associated with gp130), which in turns leads to the activation of STAT family members acting as signal transducers and activators of transcription (Calabrese & Rose-John 2014; Hirano *et al.,* 2000; Hodge *et al.,* 2005; Wang *et al.,* 2009; Weissenberger *et al.,* 2004; Yeung *et al.,* 2013).

IL-6 has been found to use an alternative pathway to activate target cells lacking the membrane-bound IL-6R via a naturally occurring soluble form of the IL-6R (sIL-6R), the so-called the IL-6 trans-signaling illustrated in Figure 4b. The membrane-bound IL-6R is cleaved by membrane-bound metalloprotease ADAM 17 to generate sIL-6R. In this pathway, the signaling is triggered by interaction of IL-6 with the sIL-6R and subsequent binding of this complex to the membrane-bound gp130 on cells that do not express the membrane-bound IL-6R. After activation, the receptor complex dimerizes and initiates the intracellular signaling. Such cells are not responsive to IL-6 when the sIL-6R is absent (Calabrese & Rose-John 2014; Yeung *et al.,* 2013).
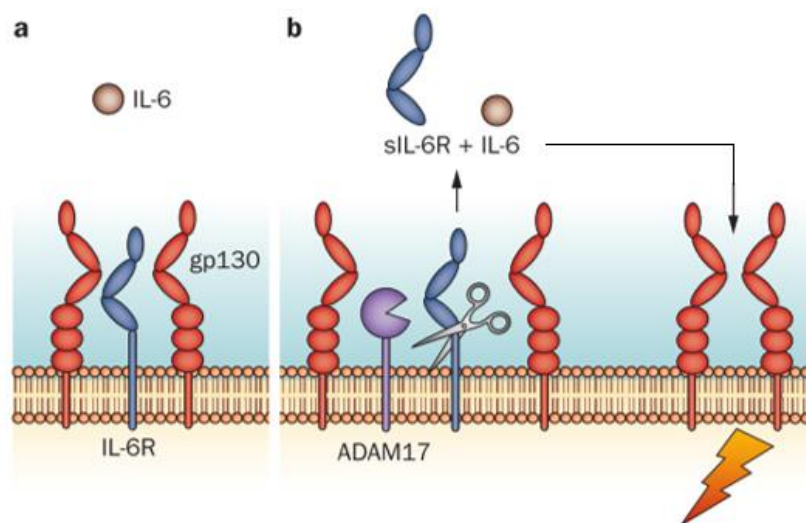
Figure 4 - Classic- and Trans-signaling of IL-6. a) In classic signaling, cells that express both IL-6R and gp130 are responsive to IL-6. b) In trans-signaling, cells that express only gp130 can be activated by the IL-6/sIL-6R complex. The sIL-6R is generated by proteolytic cleavage of the membrane bound precursor by membrane-bound metalloprotease ADAM17. (Adapted from Calabrese and Rose-John 2014).

In both pathways, after activation of the receptor, the intracellular signaling is propagated by JAK family members (JAK1-3), that leads to the activation of STAT transcription factors, namely STAT3. JAK2 can activates STAT3 by phosphorylation of the tyrosine residue in the STAT3 transactivation domain (Abou-Ghazal *et al.,* 2008). The phosphorylated STAT3 dimerizes and translocate to the nucleus where they

activate transcription factors and trigger the transcription of target genes involved in cell proliferation, apoptosis, angiogenesis, invasiveness and stemness with consequences in tumor progression (Hirano *et al.,* 2000; Hodge *et al.,* 2005; Wang *et al.,* 2009; Weissenberger *et al.,* 2004; Yeung *et al.,* 2013).

Figure 5 - Signaling pathway of IL-6 in tumors. The binding of IL-6 to the IL-6R/gp130 receptor complex leads to the activation of JAK2 that stimulates the transient activa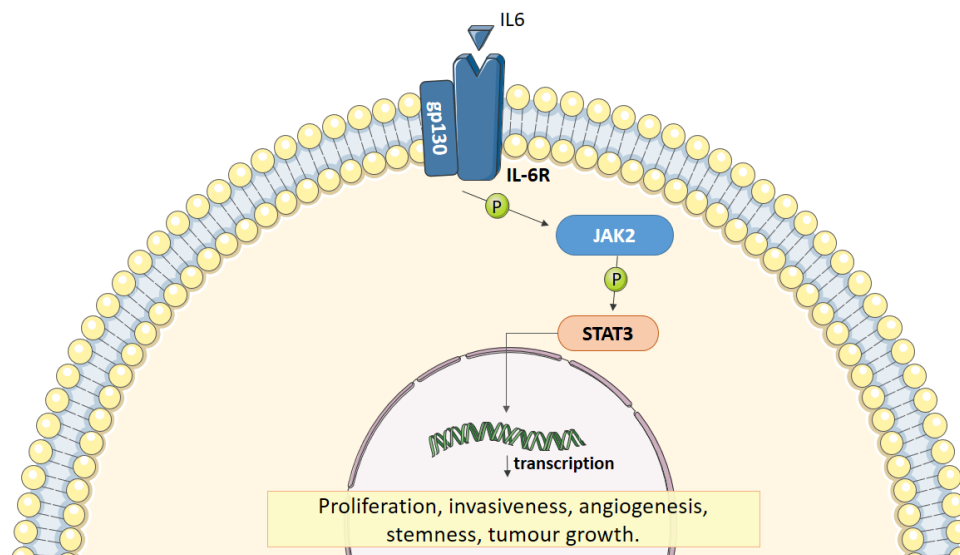tion of STAT3 transcription factor, which dimerizes and translocate to the nucleus acting as transcriptional factor of genes involved in tumor cells proliferation, invasiveness, angiogenesis and stemness properties.

STAT3 is a key transcription factor that is fundamental to control the proliferation, differentiation, development and apoptosis of normal and transformed cells (Bowman *et al.,* 2000; Hirano *et al.,* 2000; Rahaman *et al.,* 2002). Some studies show that after activation, STAT3 acts as a suppressor of apoptosis in some cancers like multiple myeloma, head and neck squamous cells carcinoma, breast carcinoma, leukemia and lymphomas (Bowman *et al.,* 2000; Rahaman *et al.,* 2002). In high-grade malignant gliomas, 60% have the STAT3 constitutively activated, and the extent of activation are positively correlated with glioma grade (Lo *et al.,* 2008). STAT3 is also an important regulator of immune suppression (Abou-Ghazal *et al.,* 2008).

The inhibition of the STAT pathway suppresses cancer cell growth and invasion, and induces apoptosis in various cancers. The inhibition of STAT3 activity through blocking the JAK may be a useful therapeutic approach against cancer. JAK2 tyrosine kinase inhibitor AG490 has been widely used as a method of blocking the

JAK/STAT3 signaling pathway, because the AG490 blocks the constitutive activation of STAT3 (Gurbuz *et al.,* 2014; Huang *et al.*, 2010).

1.5.2.2. Interleukin-6 and Glioblastoma Multiform

IL-6 is the major activator of STAT3 stimulating the formation of tyrosine-phosphorylated STAT3 (p-STAT3) in cancer cells (Huang *et al.,* 2010). The activation of STAT3 is present in glioma patient samples and increases with the tumor grade (Lo *et al.,* 2008; Rahaman *et al.,* 2002; Weissenberger *et al.,* 2004). As already mentioned, IL-6 through the JAK/STAT3 signaling pathway, promotes tumour growth and angiogenesis, protects cancer cells from apoptosis during chemotherapy and radiotherapy, and have an important role in metastasis (Hodge *et al.,* 2005; Huang *et al.,* 2010; Tchirkov *et al.,* 2007). In this pathology, IL-6 also induce the expression of VEGF, which is considered the major endothelial mitogen in glial neoplasms (Chang *et al.,* 2005; Rolhion *et al.,* 2001).

The levels of IL-6 in high-grade gliomas is higher than in low-grade gliomas, and are tightly linked to tumor progression and poor disease outcome in glioma patients. The overexpression of IL-6 is linked to tumor generation, and contributes to the malignancy, aggressiveness, bad response to therapies and is considered a poor prognostic factor in GBM patients (Wang *et al.,* 2009; Tchirkov *et al.,* 2007; Rolhion *et al.,* 2001; Loeffler *et al.,* 2005). Thereby, IL-6 is envisaged as a new potential target in the treatment of GBM, as well as a marker in glioma classification (Rolhion *et al.,* 2001; Chang *et al.,* 2005).

Objectives

2.   Objectives

It is well known that MG have an important role in neuroinflammation, a fundamental immune response to protect the CNS against pathogens and injuries. However, in a brain tumor microenvironment, MG acquire a distinct M2 activation phenotype with impaired phagocytic activity and lack of sustained anti-tumor immunity that favor tumor growth and invasiveness through the release of cytokines and other inflammatory mediators. Recent studies suggest that microglial activation and consequent neuroinflammation might be related with the dysfunction of the BBB commonly observed in several CNS diseases, including brain tumors. Despite the large amount of studies pointing out for the role of activated MG in BBB disruption, little is known about the mechanisms involved in the multifaceted interactions between MG and glioma cells, and in particular the role of the inflammatory response triggered by this interaction in BBB dysfunction.

The main goal of this project is to evaluate the effects of reciprocal interactions between MG and GBM cells in the integrity of EC monolayer.

To address this question, we propose to:

- Evaluate the effects of the interaction between MG and GBM cells, using a co-culture system, in the integrity of human EC monolayer, by measuring the TEER, permeability to different sized dextrans and structural changes in tight junction proteins;
- Evaluate the role of the inflammatory cytokine IL-6 via JAK/STAT3 pathway in the integrity of EC.

Material and Methods

3.   Material and Methods

3.1.   Cell culture

3.1.1.   Cell lines

The human glioblastoma U87 cell line and the mouse microglia BV2 cell line were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). These cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose (4 g/L; Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, 10270-106, Gibco, Invitrogen Life Technologies), 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco) at pH 7.4. The U87 and BV2 cell lines were subcultivated at a ratio of 1:10 and 1:40, respectively, twice a week when the cells reached approximately 80% of confluence. This procedure was made in sterile conditions in a laminar flow chamber.

The immortalized human brain EC line hCMEC/D3, kindly provided by Couraud (Institute Cochin, France), was derived from human temporal lobe microvessels isolated from tissue excised during a surgery for control of epilepsy. These cerebral microvessels ECs were immortalized by lentiviral vector transduction through the expression of catalytic subunit of human telomerase reverse transcriptase and SV40 large T antigen (Weksler 2005).

The hCMEC/D3 cell line was maintained in Endothelial Basal Medium-2 (EBM-2, CC-3156, Lonza, Basel, Switzerland) supplemented with 1% chemically defined lipid concentrate (Gibco, Invitrogen), 1 ng/ml^{-1} human basic fibroblast growth factor (bFGF, Sigma), 10 mM HEPES (Lonza), 1.4 µM hydrocortisone (Sigma), 5 µg/ml^{-1} ascorbic acid (Sigma), 1% penicillin-streptomycin (Gibco, Invitrogen) and 5% FBS (Merck Millipore, Biochrom, Berlin, Germany).

All cell lines were maintained in a humidified atmosphere with 5% CO_2, at 37˚C.

3.1.2. Cell viability

Cell counting and viability was determined before all experiments through the Trypan Blue exclusion method. In this method, the viable cells that have an intact and functional cellular membrane will not be stained, while the non-viable cells with a damaged cellular membrane take up the dye and appear blue under the microscope. This enables us to identify and counting viable and dead cells.

For this procedure equal volumes of cell suspension and 0.4% Trypan Blue (Sigma-Aldrich) were mixed and transferred by capillary action into a Neubauer hematocytometer chamber (Optic Labor) and then were observed and counted in an inverted microscope (Nikon, Eclipse TS 100). Both viable and non-viable cells were counted in the four corner quadrants. Cell viability were calculated as a percentage of viable cells relative to the total number of cells. Only cell suspensions with viability higher than 90% were used in all experiments.

3.1.3. Co-culture of BV2 cells with U87 cells

The co-culture of microglial (BV2) cells with glioma (U87) cells was performed using a 6-well Transwell® system (Corning Life Sciences, Tewksburg, MA, USA). The Transwell® system is composed by inserts with microporous membranes of 0.4 µm pore size. The pores allow the exchange of soluble factors released by the cells located in the upper and lower compartments as depicted in Figure 6.
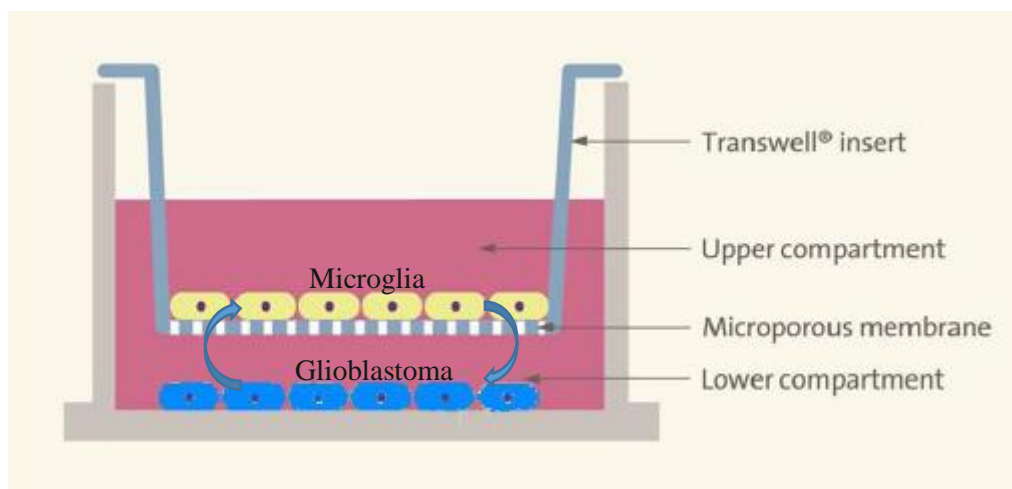

Figure 6 - Transwell system for co-culture.

U87 cells were seeded in the lower compartment at a density of 13×10^4 cells/ml and BV2 cells were plated in the bottom of the insert at a density of 6.5×10^4 cells/ml. Cells were maintained in separated wells overnight to adhere. After that the culture medium in both compartments was refreshed and the inserts containing BV2 cells were transferred to the wells with U87 cells. The co-culture system was maintained in a 5% CO_2 incubator at 37°C for 48 hours. After this period, the conditioned medium of the co-culture of BV2 with U87 (CM CC) was collected and centrifuged at 1500 rpm for 5 minutes, to remove any cell debris. Conditioned medium from individual cultures of BV2 (CM BV2) and U87 cells (CM U87) seeded at the same density as previously described, was also collected after 48 hours incubation period, and centrifuged at 1500 rpm for 5 minutes. The conditioned mediums were used in subsequent studies.

3.2.   Enzyme-Linked Immunosorbent Assay

The content of IL-6 in the conditioned media of co-cultured BV2 with U87 cells (CM CC) and of non-co-cultured cells (CM BV2 and CM U87) was evaluated using an ELISA assay (Affymetrix, eBioscience, San Diego, CA, USA) according to manufacturers' instructions.

Briefly, 96-well microtiter plates were coated with capture antibody (100 µL/well), sealed and left overnight at 4˚C. Then, wells were washed 3 times with wash buffer (0.01 M PBS plus 0.05% Tween-20) and blocked with 1x ELISA/ELISPOT diluent at room temperature (RT) for 1 hour. The samples (100 µL/well) were added to the appropriated wells and the plate was sealed and incubated for 2 hours at RT. Then, wells were aspirated and washed 5 times with wash buffer. After that 100 µL/well of the biotin- conjugated detection antibody (anti-IL-6) were added and the plate was sealed and incubated for 1 hour at RT. After washing the wells (5x), 100 µL horseradish peroxidase (HRP)-labeled avidin was added to each well and the plate was sealed and incubated for 30 minutes at RT. The wells were washed again (7x) and 100 µL/well of 1x TMB solution were added. After 15 minutes at RT, the reaction was stopped by adding 50µL of 1M phosphoric acid. The absorbance was measured in a microplate reader (Biotek, Synergy HT, Winnoski, USA), using a sample wavelength fixed at 450

nm. A standard curve for the cytokine was used to calculate the protein levels expressed in pg/ml.

3.3.   Evaluation of the integrity of the EC monolayer

Endothelial cells (hCMEC/D3) were seeded on 12-well plate transwell inserts (12 mm diameter and 0.4 µm pore size, Corning), previously coated with rat tail collagen type I (150 µg/ml; Trevigen, Cultrex, Gaithersburg, MD, US) for 1 hour, at a density of 9.2×10^4 cells/ml and were maintained in a 5% CO_2 incubator at 37°C for 3 days for endothelial monolayer formation. On third day, the culture medium was removed and replaced by conditioned mediums of the BV2-U87 co-culture (CM CC) and of non-co-cultured BV2 (CM BV2) and U87 (CM U87) cells. Non-treated hCMEC/D3 cells with conditioned mediums were used as control. To evaluate the regulatory role of IL-6 on the barrier properties of the endothelial monolayer, ECs were exposed to 10 ng/ml IL-6 (R&D Systems, Ref 406-ML/CF, UK) in the absence and in the presence of a JAK inhibitor (AG490), which is a downstream target of the IL-6 signaling pathway. For that, ECs were pre-exposed to 20 µM AG490 (Calbiochem, Ref 658401, Nottingham, UK), during 1 hour before adding 10 ng/ml IL-6 or exposure to CM CC. The inhibitor was maintained in culture during the time of the experiments.

Alterations of EC monolayer integrity, namely transendothelial electrical resistance (TEER) and permeability were performed as detailed below.

3.3.1.   Transendothelial Electrical Resistance

The TEER of the hCMEC/D3 cells monolayers was measured using a STX-2 electrode coupled to an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK). TEER readings (unit = $\Omega \cdot cm^2$) of cell-free inserts were subtracted from the total values obtained with cells, and were expressed as % of the control.

The TEER measurements were performed on third day after replacement of the cell culture medium by the aforementioned conditioned mediums at several time-points (0, 1, 2 and 4 hours) according to the experimental design shown in Figure 7. The lysophosphatidic acid (LPA, 20mM; Sigma) was used as a positive control because

increases the TJ permeability of ECs (Figure 7A) (Schulze *et al.,* 1997). To understand the role of exogenous IL-6 in the alteration of TEER, ECs were exposed to 10 ng/ml IL-6 before the measurements of TEER (Figure 7B). In another experiment cells were treated with 20 µM AG490, during 1 hour and then co-exposed with exogenous IL-6 (10 ng/ml) or co-exposed with CM CC (Figure 7C).

Figure 7 - Experimental design protocol used to measure the TEER of the hCMEC/D3 monolayer under several conditions. A. hCMEC/D3 were exposed to conditioned medium of BV2 (CM BV2), conditioned medium of U87 (CM U87) and conditioned medium of co-culture of BV2 with U87 (CM CC). LPA (20 mM), known to decrease the TJ's permeability, was used as a positive control. **B.** To understand the effect of IL-6 in the TEER of hCMEC/D3, the cells were exposed to IL-6 (10 ng/ml). **C.** The ECs were pre-treated with AG490 (20 µM), an inhibitor of the JAK2/STAT3 pathway that is activated by IL-6, before exposure to exogenous IL-6 or CM CC. The TEER was measured at several time-points (0, 1, 2 and 4 hours).

3.3.2. Permeability assay

The permeability assay allows the determination of the integrity of the monolayer of hCMEC/D3 cells, based on the measurement of the flux rate of size-selective labelled dextrans.

For these experiments, 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich) or 70 kDa Rhodamine B isothiocyanate (RITC)-dextran (Sigma-Aldrich) at a concentration of 1 mg/ml (5 µL) were added to the apical side of hCMEC/D3 grown on Transwell insert, at the same time that ECs were exposed to the experimental conditions aforementioned and illustrated in Figure 8. Samples (50 µL) were removed from the basal chamber (and replaced by correspondent conditioned medium) at 20 min intervals for 240 min. Fluorescence of samples was measured in a FLUOstar OPTIMA microplate reader (BMG LABTECH, Aylesbury, UK). The values were plotted versus time and the slope estimated by linear regression analysis.
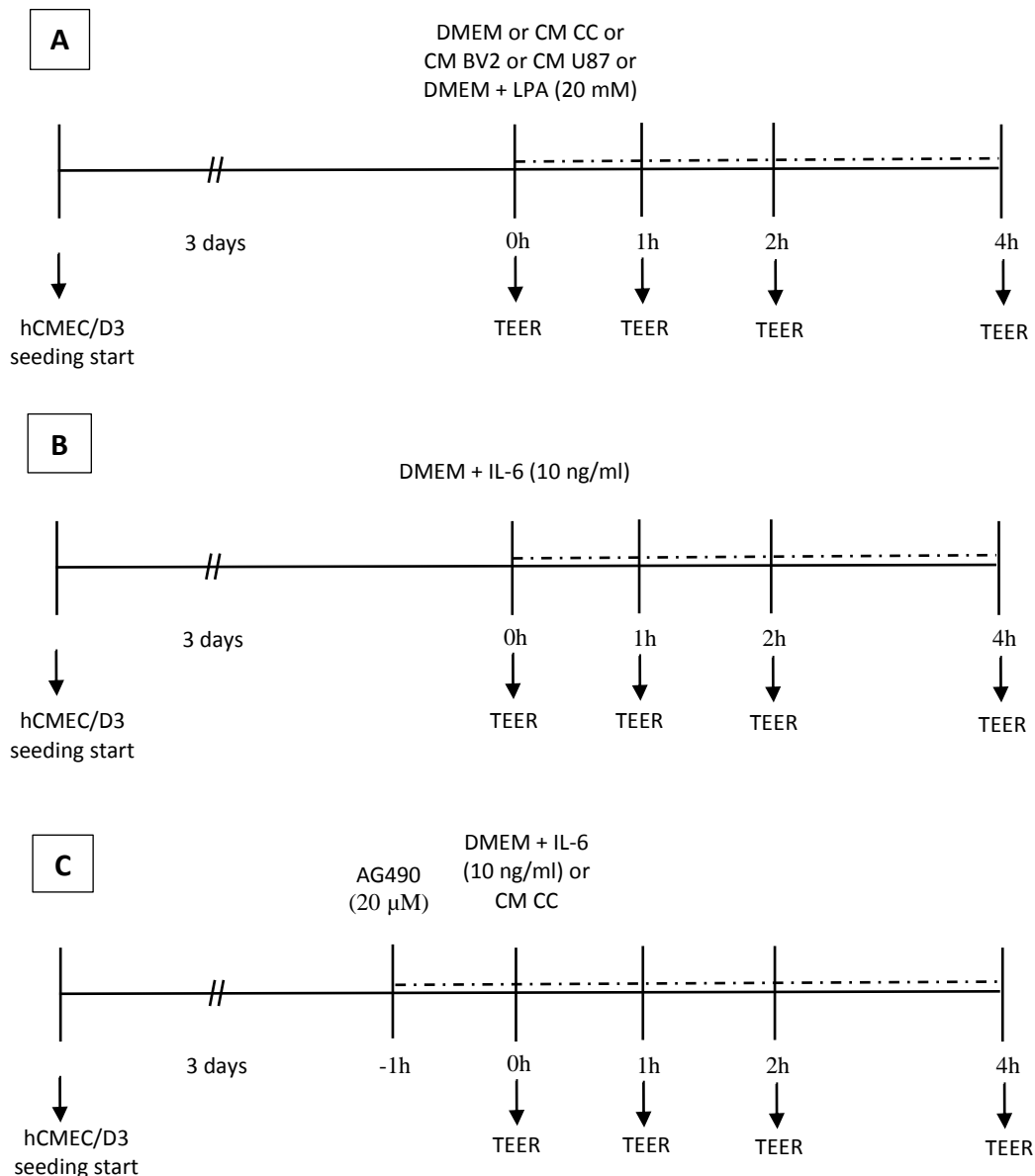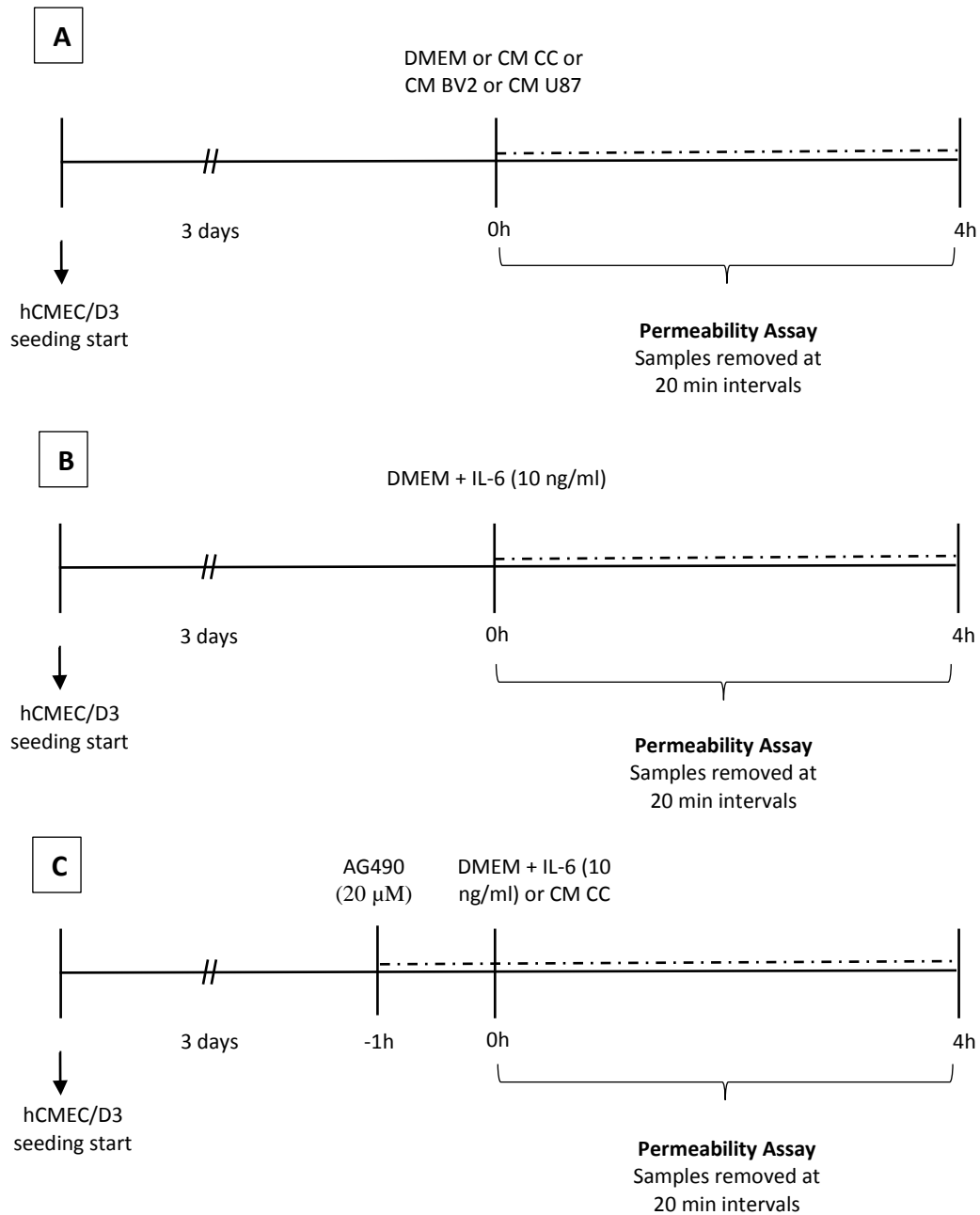
Figure 8 - Experimental design protocol used to measure the permeability of hCMEC/D3 monolayer under several conditions. **A.** hCMEC/D3 were exposed to conditioned medium of BV2 (CM BV2), conditioned medium of U87 (CM U87) and conditioned medium of co-culture of BV2 with U87 (CM CC). **B.** To understand the effect of IL-6 in the permeability of hCMEC/D3, the cells were exposed to IL-6 (10 ng/ml). **C.** The ECs were pre-treated with AG490 (20 μM), an inhibitor of JAK2/STAT3 pathway that is activated by IL-6, before exposure to exogenous IL-6 or CM CC. The permeability was measured during 4 hours, through samples removed at 20 min intervals.

3.4. Immunocytochemistry

For immunocytochemistry analysis of claudin-5 and ZO-1, hCMEC/D3 cells were seeded in coverslips, previously coated with rat tail collagen type I (300 µg/ml; Trevigen®, Cultrex®) for 1 hour, at a density of 18.4×10^4 cells/coverslip and maintained in a 5% CO_2 incubator at 37ºC until endothelial monolayer formation. Thereafter, EC were exposed to fresh DMEM culture medium (control), CM CC, CM CC + 20 µM AG490 and DMEM + 10 ng/ml IL-6, for 2 hours.

For claudin-5 analysis, the ECs were fixed with methanol for 15 min at -20˚C. After fixed, cells were washed 3 times with 1x PBS and then permeabilized with 0.1% Triton-X for 10 min at RT and blocked with 5% Bovine Serum Albumin (BSA) during 45 minutes at RT.

For ZO-1 analysis, the ECs were fixed with 4% Paraformaldehyde (PFA) for 20 minutes at RT. After fixed, cells were washed 3 times with 1x PBS and then permeabilized with 0.2% Triton-X for 3 min at RT and blocked with 3% BSA during 1 hour at RT.

Afterwards, cells were incubated with the primary antibody to claudin-5 (1:50; Invitrogen™) and ZO-1 (1:100; Invitrogen™, Zymed), overnight at 4˚C. After cells were incubated with secondary antibody Alexa Fluor 488 anti-mouse (1:200; Invitrogen™) or Alexa Fluor 488 anti-rabbit (1:200; Invitrogen™), respectively. Cells were washed again with 1xPBS 3 times and stained with Hoechst (stock 2mg/ml) (1:1000; Sigma-Aldrich®) for 5 minutes at RT in the dark. Finally, cultures were mounted in Dako fluorescence medium (Dako North America Inc.) and images were captured using a LSM 710 Meta confocal microscope (Carl Zeiss, Göttingen, Germany).

3.5. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using the multiple level analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test (GraphPad Software, San Diego, CA, USA).

Results

4.   Results

4.1.   The integrity of the EC monolayer is not affected by DMEM culture medium

The EC hCMEC/D3, used as a BBB model, grow and were maintained in EBM-2 medium, whereas microglial and glioma cells were kept in DMEM medium. Since during our experiments the hCMEC/D3 cells were exposed to DMEM medium collected from the BV2/U87 co-cultures, we started to evaluate if the DMEM medium *per se* induced any alteration on the integrity of the EC monolayer.

To address the potential influence of DMEM in the alteration of the EC monolayer integrity, we measured the TEER and the permeability of 4 kDa FITC dextran of the hCMEC/D3 monolayer after replacement of the EBM-2 culture medium by DMEM. This assay was performed 3 days after plating hCMEC/D3 cells in transwell inserts for an efficient formation of the monolayer.

The TEER was measured immediately before the replacement of the culture medium which corresponds to t=0 and at 1, 2 and 4 hours after exposure to the DMEM medium. The values in each experimental condition were normalized to those measured at t=0.
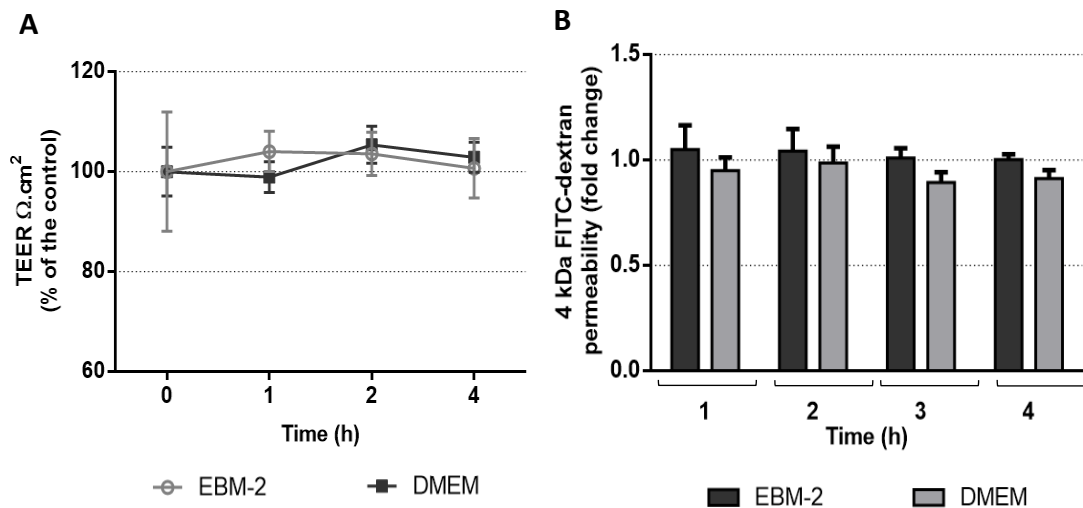
Figure 9 - Effect of DMEM on endothelial cells monolayer integrity. The EC integrity was evaluated by measuring TEER (A) and 4 kDa FITC-dextran permeability (B) at different time points after DMEM exposure. All results are shown as means ± SEM, n=3.

The permeability assay was performed during 4 hours using a 4 kDa FITC-dextran. Samples were collected in intervals of 20 minutes from the chamber below the EC monolayer.

No significant changes were observed in the TEER (Figure 9A) nor in the 4 kDa FITC-dextran permeability (Figure 9B) of the EC monolayer after changing regular EBM-2 culture medium by DMEM. These results showed that DMEM, by itself, does not promote changes on the EC monolayer integrity, namely alteration in TEER or in the 4kDa FITC-dextran permeability at least during the 4 hours of the experiment.

4.2. The bi-directional interactions between MG and glioma cells changes the TEER and the permeability of the EC monolayer

Since the main goal of this work was to investigate the effects of the interaction between MG and tumor cells in the integrity of EC, we evaluated the effects of the conditioned medium collected directly from co-cultures of BV2 with U87 cells in the barrier properties of the hCMEC/D3 cells monolayer. We measured two parameters: the TEER as indicator of TJ permeability and the permeability to a low (4 kDa) and high (70 kDa) molecular weight dextrans to quantify the paracellular flux across the cell monolayer.

To evaluate the individual contribution of MG and glioma cells on the EC monolayer integrity, we also used conditioned mediums of BV2 and U87 cells under non-co-cultured conditions. All conditioned mediums were collected at 48 hours. Fresh DMEM culture medium was used as a control to assess the integrity of EC monolayer on normal conditions. LPA was used as a positive control because it promotes a rapid, reversible and concentration-dependent decrease of the TEER together with an increase in the paracellular flux of sucrose through brain EC, indicating increased TJ permeability (Schulze *et al.,* 1997).

The TEER measurements were performed immediately before (t=0) and at 1, 2 and 4 hours after exposure the EC monolayer to the different conditioned mediums. Each experimental condition has its own control which corresponds to the TEER values measured on time 0. The results were expressed as percent of the control.
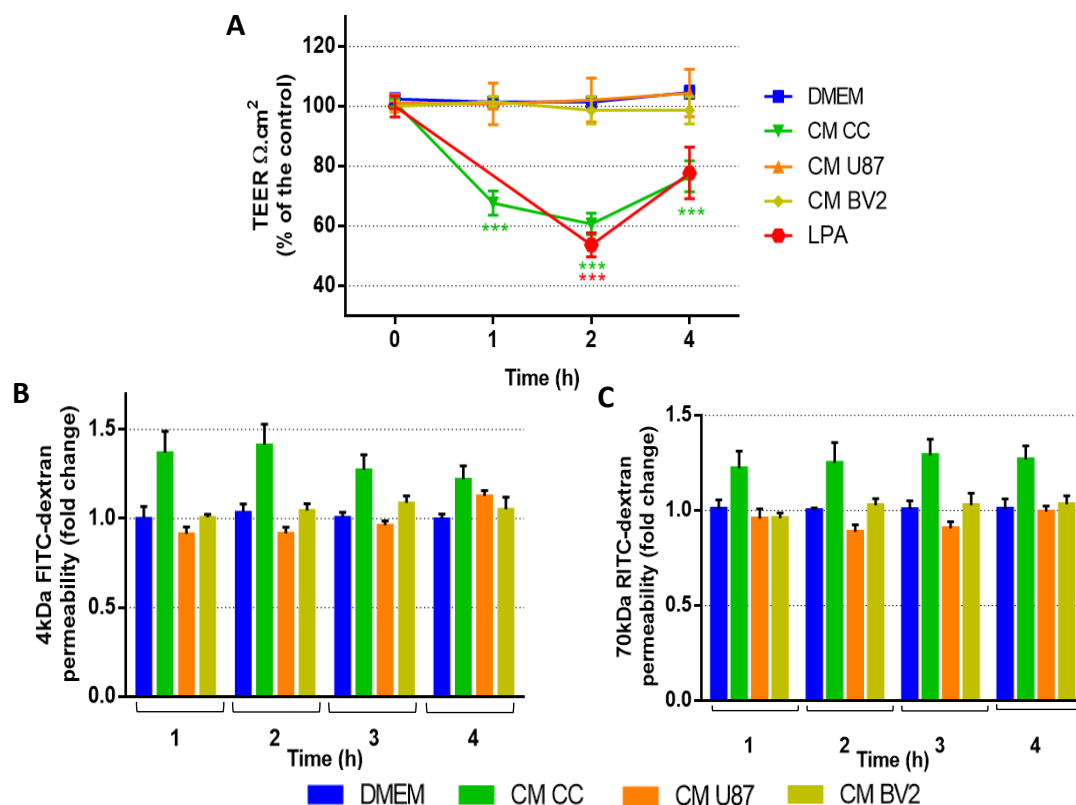
Figure 10 - Effect of conditioned mediums on endothelial cells monolayer integrity. The EC integrity was evaluated by TEER (A) and permeability assays with 4 kDa FITC (B) and 70 kDa RITC (C) at different time points after CM CC, CM U87, CM BV2 and LPA exposure. All results are shown as means ± SEM, n= 3 to 12. One-way ANOVA analysis followed by Bonferroni's post-test was used for multiple comparisons ***P<0.001, compared with the DMEM-treated control cells.

Results

When EC monolayer was exposed to CM CC (n=12) it was observed a significant decrease on the TEER values at all time-points measured in relation to those measured at t=0 (Figure 10A). During the first hour, the TEER decreased to 67.67±4.03% and continued to decline reaching the 60.71±3.62% after the second hour. At 4 hour it was observed a partial recovery of the TEER to 76.61±5.17% (n=12). No significant changes were observed in the TEER of EC monolayer exposed to CM U87 (n=7) or CM BV2 (n=3) in relation to the DMEM-treated control cells. The exposure of EC monolayer to LPA (20 mM, n=8) showed, as expected, a significant decrease in the TEER after 2 hour (53.71±4.03, p<0.001) and 4 hour (77.74±8.64), similar to that induced by the CM CC. The TEER values are summarized in Table 1.

Table 1 - Effects of conditioned mediums in TEER of endothelial cells monolayer.

	0h	1h	2h	4h
DMEM	102.48 ± 1.73 %	101.46 ± 0.93%	101.47 ± 1.03 %	104.75 ± 2.02 %
CM CC	101.35 ± 1.44 %	67.67 ± 4.03 %[***]	60.71 ± 3.62 %[***]	76.61 ± 5.17 %[***]
CM U87	101.10 ± 2.83 %	100.83 ± 6.94 %	102.11 ± 7.32 %	104.45 ± 7.93 %
CM BV2	100.00 ± 1.88 %	101.61 ± 1.61 %	98.672 ± 4.55 %	98.67 ± 4.55 %
LPA	100.00 ± 3.52 %	-	53.71 ± 4.03 %[***]	77.74 ± 8.64 %

TEER values were normalized to respective initial values (t=0) for each experimental condition and were expressed as mean ± SEM (n=3 to n=12). One-way ANOVA analysis followed by Bonferroni's post-test was used for multiple comparisons. ***P<0.001, compared with the DMEM-treated control cells of each time point.

The permeability assay was performed during 4 hours using a 4 kDa FITC-dextran (Figure 10B) and a 70 kDa RITC-dextran (Figure 10C) that were added on the apical area of the EC monolayer. This assay allows us to quantify the paracellular flux of the EC monolayer to the different-size dextrans used. The samples were collected in intervals of 20 minutes from the chamber below the EC monolayer, allowing us to quantify the amount of dextrans that have passed through the EC monolayer. We used 2 different-size dextrans to estimate the degree of damage induced by CM CC on the barrier properties of the EC monolayer.

Exposure to CM CC increased, although not statistically significant, the permeability of the EC monolayer to two dextrans 4 kDa-FITC and 70 kDa-RITC, reaching a maximum at 2 hour, as shown in Figure 10B. None of the collected conditioned media of MG or tumor cells induced any alterations on the permeability of

the EC monolayer to 4 and 70 kDa dextrans, as observed with TEER. The permeability to 4 and 70 KDa dextrans expressed as fold-changes relative to control, and under the different experimental conditions, are shown in Tables 2 and 3 respectively.

Table 2 - Effects of conditioned mediums on endothelial cells monolayer permeability to 4 kDa FITC-dextran.

	1h	2h	3h	4h
DMEM	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
CM CC	1.37 ± 0.12	1.41 ± 0.12	1.27 ± 0.09	1.22 ± 0.08
CM U87	0.91 ± 0.04	0.92 ± 0.04	0.96 ± 0.02	1.13 ± 0.03
CM BV2	1.01 ± 0.02	1.04 ± 0.04	1.09 ± 0.04	1.05 ± 0.07

Table 3 - Effects of conditioned mediums on endothelial cells monolayer permeability to 70 kDa RITC-dextran.

	1h	2h	3h	4h
DMEM	1.00 ±0.05	1.00 ± 0.01	1.01 ±0.04	1.01 ± 0.05
CM CC	1.22 ± 0.09	1.25 ± 0.11	1.29 ± 0.08	1.27 ± 0.07
CM U87	0.95 ± 0.05	0.89 ± 0.04	0.91 ± 0.03	0.99 ± 0.03
CM BV2	0.95 ± 0.03	1.03 ± 0.03	1.03 ± 0.06	1.03 ± 0.05

4.3. The bi-directional interactions between BV2 and U87 cells promotes the release of IL-6

After verifying that CM CC affects the integrity of EC monolayer, we decided to identify which factor released in the conditioned medium of the co-culture can induce this effect. In a previous study performed in our laboratory, the analysis of cytokines mRNA expression by real-time PCR in MG-GBM co-cultures showed a pronounced and consistent upregulation of IL-6 in relation to the constitutive levels found in non-co-cultured cells.

Taking into account these results and the fact that IL-6 activates the JAK-STAT pathway that are linked to proliferation, invasiveness, angiogenesis and tumor cells growth (Yeung *et al.,* 2013) we decided to study the role of IL-6 on disruption of the EC monolayer.

We performed an ELISA assay to measure the amount of IL-6 cytokine released in the conditioned mediums of co-cultures (CM CC) and of the individual cells (CM BV2 and CM U87). Fresh DMEM was used as a negative control.
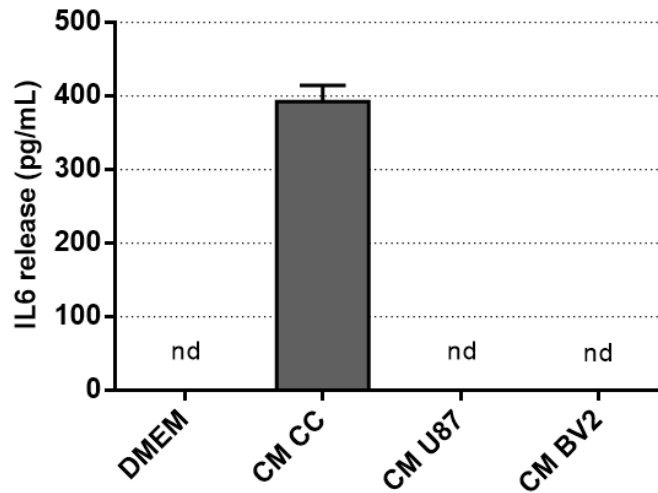

Figure 11 - Content of IL-6 in conditioned mediums of co-culture and non-co-cultured BV2 and U87 cells. The levels of IL-6 were evaluated by ELISA in conditioned mediums collected after 48 hours of culture. Legend: nd, not detectable.

The ELISA analysis showed high levels of IL-6 (392.2±22.27 pg/ml) in the conditioned medium of BV2/U87 co-culture. No detectable levels were found on the conditioned mediums of BV2 or U87 cells cultured individually neither in fresh DMEM medium (Figure 11).

4.4. IL-6-mediated disruption of EC monolayer is prevented by inhibition of JAK/STAT3 pathway

To clarify the role of IL-6 in the alterations observed in the EC monolayer exposed to CM CC, we perform the TEER and permeability assays in the presence of exogenous IL-6, trying to understand if in the presence of IL-6 alone we can observe similar results to obtained with CM CC. These assays were also performed in the presence of AG490, an inhibitor of JAK/STAT3 pathway that is activated downstream by IL-6 (Yeung *et al.,* 2013)*.* By this way, a recovery on the TEER and permeability values in the presence of the inhibitor AG490, provides evidence for a pivotal role of IL-6 in disruption of the EC monolayer integrity by activation of the JAK/STAT3 pathway.

To confirm this hypothesis, the TEER and permeability assays were performed with EC monolayer exposed to DMEM containing 10 ng/ml IL-6 or 10 ng/ml IL-6 + 20 µM AG490, and to CM CC with and without 20 µM AG490. Fresh DMEM culture medium was used as a control.
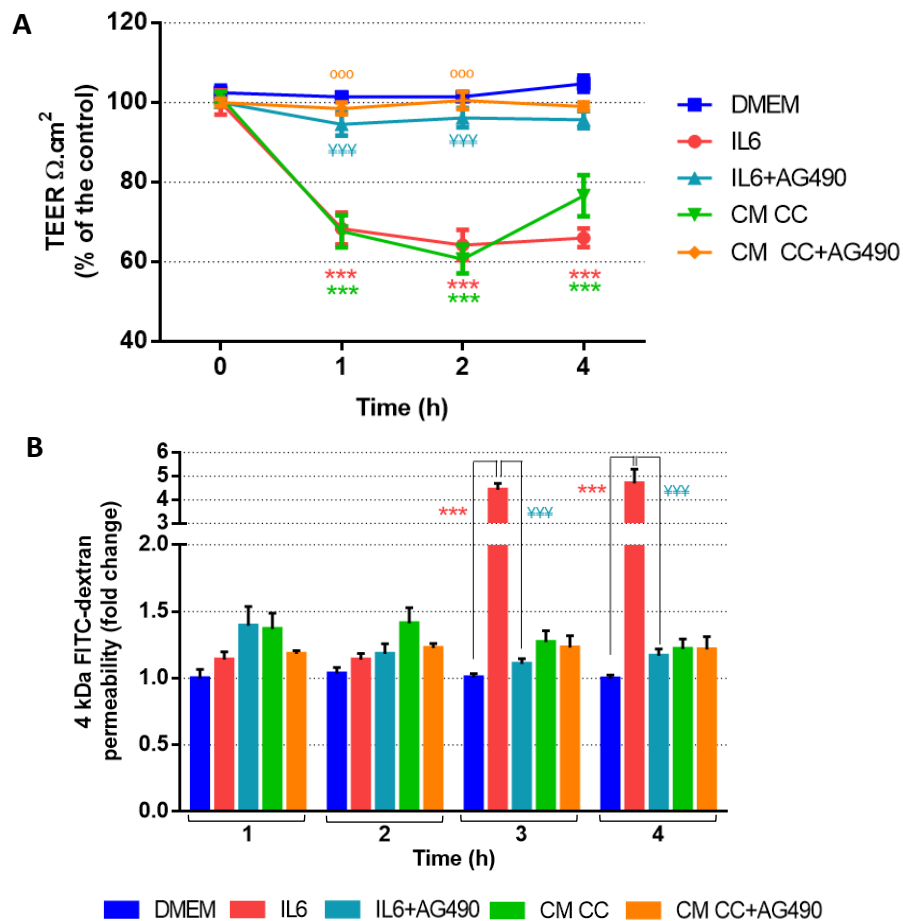
Figure 12 - Effects of IL-6 in endothelial cell monolayer TEER and permeability. The EC integrity was evaluated by measuring the TEER (A) and 4 kDa FITC permeability (B) at different time points during 4 hours. EC monolayer were exposed to 10 ng/ml IL-6 and CM CC in the absence and in the presence of 20 µM AG490. Results are presented as mean ± SEM, (n=3 to 12). One-way ANOVA analysis followed by Bonferroni's post-test was used for multiple comparisons ***$P < 0.001$, compared with DMEM-treated control cells at each time point. ¥¥¥$P < 0.001$, IL-6 compared with IL-6 + AG490 treated cells. ooo$P < 0.001$ CM CC compared with CM CC + AG490 treated cells.

The exposure of EC monolayer to 10 ng/ml of IL-6 (n=4) induced a significant decrease in the TEER assay similar to that induced by CM CC as depicted in Figure 12A. The addition of the JAK inhibitor AG490 (20 µM) prevented the IL-6-mediated TEER decrease in EC monolayer exposed to CM CC or exogenous IL-6. The mean values of TEER normalized to respective controls are shown in table 4.

Results

Table 4 - Effects of IL-6 in TEER of endothelial cells monolayer.

	0h	1h	2h	4h
DMEM	102.48 ± 1.73	101.46 ± 0.93	101.47 ± 1.03	104.75 ± 2.02
IL-6	100.00 ± 3.00	68.35 ± 3.99***	64.21 ± 3.79***	66.03 ± 2.36***
IL-6 + AG490	100.00 ± 0.83	94.58 ± 2.92¥¥¥	96.18 ± 2.431¥¥¥	95.69 ± 2.16
CM CC	101.40 ± 1.44	67.67 ± 4.03***	60.71 ± 3.62***	76.61 ± 5.17***
CM CC + AG490	100.00 ± 0.99	98.53 ± 1.47°°°	100.5 ± 2.18 °°°	99.02 ± 0.98

TEER values were normalized to respective initial values (t=0) for each experimental condition and were expressed as mean ± SEM (n=3 to n=12). One-way ANOVA analysis followed by Bonferroni's post-test was used for multiple comparisons. **$P<0.01$, ***$P<0.001$, compared with DMEM-treated control cells of each time point. °°°$P<0.001$, CM CC compared with CM CC + AG490 treated cells. ¥¥¥$P<0.001$, IL-6 compared with IL-6 + AG490 treated cells.

In the permeability assay we only tested the 4 kDa dextran, since the most pronounced effects induced by CM CC were observed for this hydrophilic molecule.

The addition of 10 ng/ml IL-6 induced a pronounced and significant increase in the permeability of the EC monolayer after 3 and 4 hours of incubation, an effect that was abolished by the presence of AG490, as depicted in Figure 12B. The addition of AG490 also prevented the increase in endothelial permeability induced by the CM CC at 1 and 2 hours. The mean values of permeability coefficients are presented in Table 5.

Table 5 - Effects of IL-6 on endothelial cells monolayer permeability to 4 kDa FITC-dextran.

	1h	2h	3h	4h
DMEM	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
IL-6	1.14 ±0.06	1.11 ± 0.05	4.42 ± 0.27***	4.69 ± 0.60***
IL-6 + AG490	1.40 ± 0.15	1.18 ± 0.08	1.11 ± 0.04¥¥¥	1.17 ± 0.05¥¥¥
CM CC	1.37 ± 0.12	1.41 ± 0.15	1.27 ± 0.09	1.22 ± 0.08
CM CC + AG490	1.18 ± 0.03	1.23 ± 0.03	1.23 ± 0.09	1.22 ± 0.10

Permeability values are expressed as a mean of fold change ± SEM (n=3 to n=12). One-way ANOVA analysis followed by Bonferroni's post-test was used for multiple comparisons. ***$P<0.001$ compared with the DMEM-treated control cells. ¥¥¥$P<0.001$, IL-6 compared with IL-6+AG490 treated cells.

4.5.   IL-6 decreased the expression of TJ proteins claudin-5 and ZO-1 via activation of the JAK/STAT pathway.

After verifying that the IL-6 released in the CM CC induced alterations in the barrier properties of the EC monolayer, next we analyzed the expression and distribution of intercellular junction proteins by immunohistochemistry, specifically the TJ proteins claudin-5 and ZO-1. The TJ are located in the luminal side of the lateral plasma membrane and regulates lateral diffusion between the luminal and abluminal plasma membrane, controlling the permeability of the paracellular space.

Exposure of EC monolayer to CM CC induced a significant decrease in the expression and changes in the cellular distribution of claudin-5 and ZO-1, similar to that induced by exogenous IL-6 (10 ng/ml), as depicted in Figure 13.

Claudin-5, which under control conditions, (Figure 13A), display strong immunoreactivity along the plasma membrane of adjacent cells, appears less intense and more diffuse in treated cell with CM CC or IL-6. Also, ZO-1 located in the cytoplasmic side of TJ between neighboring cells, decreased after exposure to CM CC or IL-6.

To determine whether this effect of IL-6 on TJ proteins was dependent on the activation of the JAK/STAT pathway, we block this pathway with AG490 (20 μM). The inhibition of JAK/STAT pathway prevented the downregulation and changes in cellular distribution of claudin-5 and ZO-1 induced by the IL-6 content in the CM CC (Figure 13). Altogether these results provide evidence that IL-6 downregulated the expression of TJ proteins via activation of the JAK/STAT3 pathway.
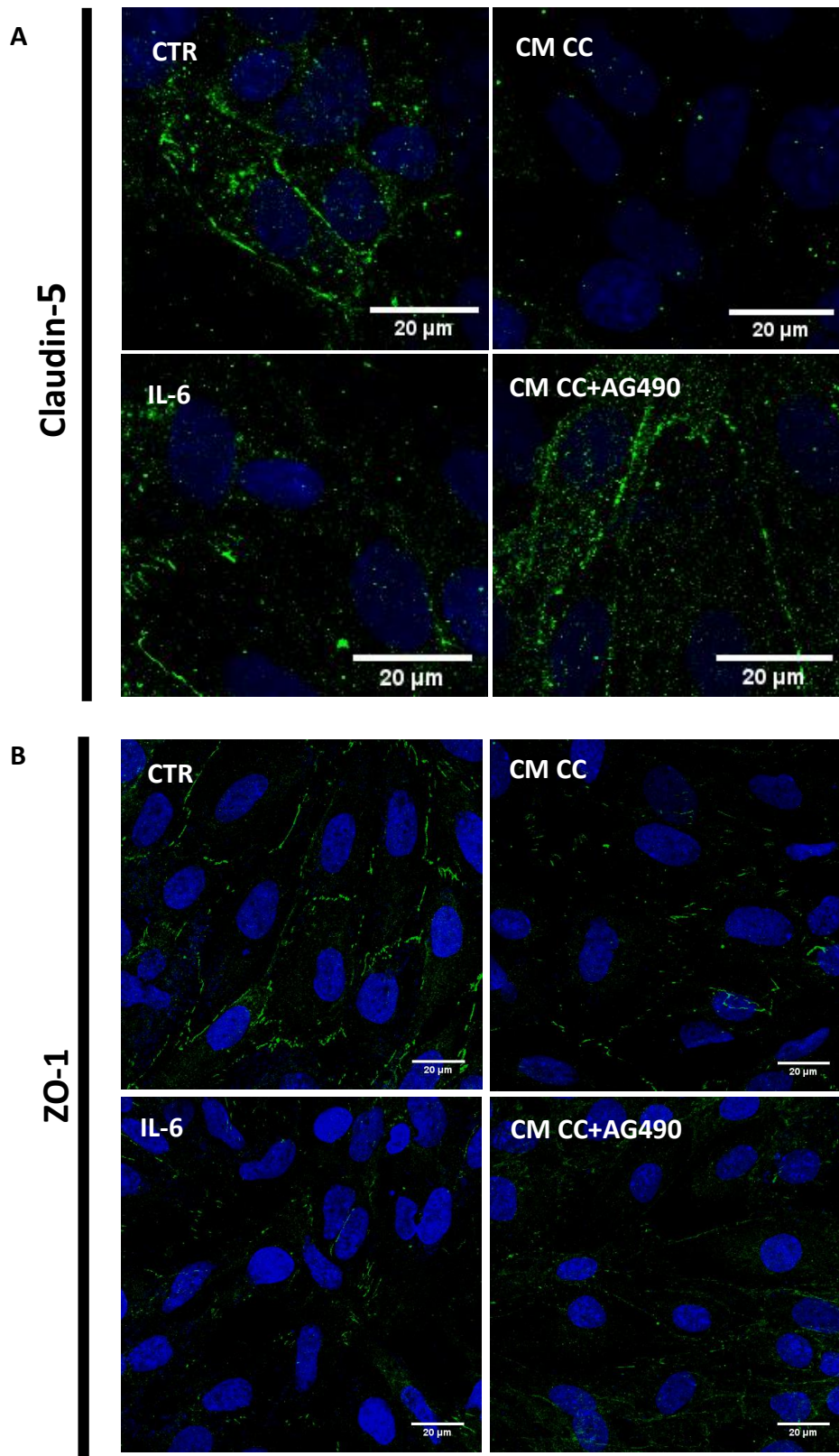
Figure 13 - IL-6 decreased the expression of tight junction proteins Claudin-5 and ZO-1 in endothelial cells monolayer. Representative confocal images of claudin-5 (A) and ZO-1 (B) immunoreactivity in EC under different experimental conditions as follow: untreated (Ctr); CM CC; 10 ng/ml IL-6 and CM CC + 20 µM AG490. Claudin-5 and ZO-1 (green). Nuclei were stained with Hoechst 33342 (blue). Scale bar 20 µm (63x magnification).

Discussion

5.   Discussion

MG has an important role in neuroinflammation acting as the first and main form of active immune defense in the CNS. The activation state of MG is highly influenced by the surrounding microenvironment (Kofler & Wiley 2011). Several studies suggest that in the glioma microenvironment, MG acquired a distinct M2 activation phenotype and release a wide variety of growth factors and cytokines that promotes tumor proliferation, survival and invasiveness (Li & Graeber 2012). GBM is highly vascularized to ensure survival and continuous tumor growth, and is characterized by microvascular proliferation and a highly abnormal dysfunctional vasculature with negative effects for patients. The new vessels formed from pre-existing vasculature have morphological alterations, such as formation of fenestrations and TJ's disruption with consequent abnormal function (Dubois *et al.,* 2014). Neuroinflammation has long been known to disrupt the integrity of BBB. Multiple studies suggest that MG under influence of tumor cells triggers a pronounced inflammatory response characterized by release of cytokines, chemokines and signal transduction molecules with impact in the BBB properties. However, the mechanisms underlying this effect are not completely clarified. In this study we analyzed how the bi-directional interactions between MG and GBM cells, through release of soluble factors, affected the integrity of the EC monolayer.

We used a human GBM U87 cell line, a mouse MG BV2 cell line and a human brain EC line hCMEC/D3, that was used as a model of BBB. To simulate the neuroinflammatory response resulting from the dynamic crosstalk between MG and GBM cells, we used a MG-GBM co-culture system in transwells, which allows the bi-directional diffusion of soluble factors. Before starting the studies, we analyzed, by measuring the TEER and 4 kDa permeability of the EC monolayer, whether the DMEM medium *per se* had any effect on EC monolayer, since this is not the recommended medium for culturing EC. No significant alterations were observed with the DMEM medium *per se* so we move forward with subsequent studies.

Discussion

The exposure of EC monolayer to conditioned medium of theMG-GBM co-culture induced significant alterations in the permeability of EC monolayer, as demonstrated by decreased TEER and augmented paracellular permeability to 4 kDa and 70 kDa-dextrans. The TEER measurements and permeability to hydrophilic dextrans provide information relative to the formation of fenestrations and TJ disruption, since an increase in the number of open pores between adjacent EC will be manifested as a decrease in electrical resistance across the EC monolayer, together with an increase in the paracellular flux of dextrans across the EC monolayer. The fact we had observed an increase in the permeability of EC to either a low (4 kDa) and a high (70 kDa) molecular weight dextran confirmed the damages in the integrity and barrier function of EC. We also measured the individual contribution of MG and GBM cells on the EC monolayer integrity through the exposure of conditioned mediums of BV2 and U87 cells under non-co-cultured conditions and no effects on EC monolayer were measured, indicating that the disturbances in EC are mediated by soluble factors released during the MG-tumor cell interactions.

These alterations on the permeability of EC monolayer were accompanied by alterations on the TJ structure and organization that also affect the permeability of the BBB. The exposure of EC monolayer to conditioned medium of MG-GBM co-culture induced a significant decrease in the expression and changes in the cellular distribution of claudin-5 and ZO-1 that are important elements to TJ structure and responsible for the adhesion of one EC to another. Changes in the cellular distribution of these proteins leads to an increase in the paracellular permeability of EC with negative impact in BBB permeability.

Inflammatory brain diseases, such as multiple sclerosis or bacterial meningitis, can induce changes in the integrity of the BBB, through the cytokines that are released after injury or inflammation. Hoffman and colleagues have shown, in brain edema associated with diabetic ketoacidosis, that inflammatory mediators have a potential involvement in BBB breakdown. Through the measuring of TEER they saw that the exposure to TNF, IL-1β and IL-6, cytokines involved in various pathologies, leads to a decrease of TEER of EC indicating a disruption of BBB. These disruption was manifested

by the absence of TJ's proteins (such as, occludin, claudin-5, ZO-1 and JAM-1) in EC (Hoffman *et al.,* 2009).

Elevated levels of IL-6 have been identified in a variety of systemic inflammatory diseases and are associated with endothelial barrier dysfunction, however the specific effect of IL-6 on endothelial permeability as well the mechanism of action have not been fully examined (Maruo *et al.,* 1992).

The analysis of IL-6 by ELISA showed a pronounced increase in the secretion of this cytokine in the conditioned medium of the co-culture, but not in the conditioned mediums of non-co-cultured cells whose levels were not even detected, suggesting that GBM cells lead microglial cells to produce IL-6. Similar results were reported by Zhang and colleagues that also found high levels of IL-6 in the co-culture of MG with GBM cells (Zhang *et al.,* 2012). Taking these results into account and knowing that IL-6 interferes with the permeability of BBB, we decided to explore if this cytokine is really involved in the dysfunction of EC cells monolayer that we previously observed.

The exposure of EC monolayer to exogenous IL-6 induced pronounced alterations in the integrity of EC monolayer, namely decreased TEER and increased 4 kDa FITC-dextran permeability as well as changes in the cellular distribution of claudin-5 and ZO-1 similar to those induced by CM CC. De Vries and colleagues also observed a gradual decrease in the TEER of EC after exposure to exogenous IL-6 (De Vries *et al.,* 1996). In another study, Maruo and colleagues studied the role of IL-6 in endothelial permeability in inflammatory diseases, such as rheumatoid arthritis. They observed a remarkable increase in the permeability of fluorescein isothiocyanate-labeled albumin across the bovine vascular EC after treatment with 100 ng/ml IL-6 during 21 hours, an effect that was completely abolished by the addition of anti-IL-6 antibody. The light microscopic observation of a cross-section of a monolayer revealed changes in cell shape and rearrangement of intracellular actin fibers, which correlates with the increased permeability induced by IL-6 (Maruo *et al.,* 1992). The reversible changes on endothelial permeability induced by hypoxia is also mediated by IL-6. According to this study, the generation of ROS during hypoxia act as signaling elements and regulates

the secretion of IL-6 that lead to alterations of endothelial permeability in HUVEC cells (Ali *et al.,* 1999).

These findings, along with our results suggest that IL-6 is an important mediator of increased endothelial permeability via alterations in the ultrastructural distribution of TJ. We hypothesized that this effect occurs via the JAK/STAT signaling pathway, which is activated by inflammatory cytokines including IL-6. The JAK/STAT pathway plays a prominent role in cytokine-mediated inflammatory responses, and as such has been implicated in EC dysfunction (Yeung *et al.,* 2013). We then investigated the role of JAK/STAT pathway in IL-6-induced EC dysfunction, by using AG490, a JAK specific inhibitor that blocks the constitutive activation of STAT3. Likewise, the inhibition of the JAK/STAT pathway with AG490 prevented the EC dysfunction induced by the CM CC or exogenous IL-6. Indeed, the AG490 was able to prevent the increase in endothelial permeability to small molecular weights dextrans as well as the decrease in TEER. Accordingly, no changes were observed in the expression and organization of TJ's proteins (claudin-5 and ZO-1).

Overall, in this work we demonstrated that MG under the influence of GBM cells, impaired the integrity of the EC monolayer by disrupting of TJ, via IL-6-induced JAK/STAT pathway. These results provide new insights into the mechanisms underlying the disruption of BBB permeability in GBM.

Conclusions

6.   Conclusions

Based on the results obtained in this study, we conclude:

- The dynamic interactions between MG and GBM cells creates an inflammatory environment that disrupt EC monolayer integrity, resulting in decreased TEER, increased 4 kDa and 70 kDa-dextrans permeability and changes in the cellular distribution of claudin-5 and ZO-1;

- MG under the influence of GBM cells, impaired the integrity of the EC monolayer by disrupting of TJ, through the release of IL-6 and subsequent activation of the JAK/STAT pathway;

- The blockade of IL-6 activity via inhibition of JAK/STAT pathway with AG490, prevented the EC monolayer dysfunction.

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