Targeting the anti-inflammatory interplay promoting glioblastoma progression with combined natural killer cells and mab9.2.27 against NG2/CSPG4

A novel strategy to reveal glioblastoma to the immune system

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SCIENTIFIC ENVIRONNEMENT

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TABLE OF CONTENTES

ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AIM	Absent In Melanoma
ALM	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APC	Antigen Presenting Cells
a-SMA	alpha-Smooth Muscle Actin
At	Astatine
BBB	Blood Brain Barrier
BCSFB	Blood CSF Barrier
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
CARs	Chimeric Antigen Receptors
CD	Cluster of Differentiation
CDC	Complement Derived Cytotoxicity
CED	Convection Enhanced Delivery
CML	Chronic Myeloid Leukemia
CMV	Cytomegalovirus
CNS	Central Nervous System
CPC	Cancer Progenitor Cells
CpG-ODNs	Oligodeoxynucleotides Containing CpG motifs
CSC	Cancer Stem Cells
CS-GAG	Chondroitin Sulfate Glycosaminoglycan
CT	Computer Tomography
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cells
EGF	Endothelial Growth Factor
EPC	Endothelial Progenitor Cells
EphA2	Erythropoietin-producing hepatocellular carcinoma A2
FDG	Fluorodeoxyglucose
GAA	Glioma Associated Antigens
GBM	Glioblastoma
GDNF	Glial Cell-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
Gp100	Human melanoma-associated antigen
GRIP	Glutamate Receptor Interaction Protein
GSC	Glioma Stem Cells
HER-2/neu	Human Epidermal Growth Factor Receptor 2
HIF-1a	Hypoxia Inducible Factor-1 alpha
HLA	Human Leukocyte Antigen

HSC	Hematopoietic Stem Cell
I	Iodine
IDH1/2	Isocitrate Dehydrogenase 1 and 2
IFN	Interferon
IL	Interleukin
IP	Intraperitoneal
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
LAK	Lymphokine-Activated Killer
LC	Liposome Clodronate
LG	Laminin G
LI	Labeling Indices
LNS	Laminin G / Neurexin/ Sex Hormone Binding Globulin
LPS	Lipopolysaccharide
mAb	monoclonal Antibody
MAGE	Melanoma-Associated Antigen
MCP	Monocyte Chemoattractant Protein
MCSP	Melanoma Cell Surface Proteoglycan
MGMT	O6-methylguanine-DNA methyltransferase
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteases
MRI	Magnetic Resonance Imaging
MUPP1	Multi-PDZ Domain Protein 1
NG2/*CSPG4*	Neuron Glia 2/Chondroitin Sulphate Proteoglycan 4
NK	Natural Killer
NPB	Non Pathological Brain
NSC	Neural Stem Cells
OPC	Oligodendrocyte Precursor Cells
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cell
PDGF-β	Platelet Derived Growth Factor-beta
PE	Pseudomonas Exotoxin
PET	Positron Emission Tomography
PFS	Progression-Free Survival
PGE2	Prostaglandin E2
SART	Squamous Cell Carcinoma Antigen Recognized by T Cells
SDF-1	Stromal Cell-Derived Factor-1
SE	Spin-Echo
SOX	Sry-Related High-Mobility Group Box
TGF	Tumor Growth Factor
TIL	Tumor Infiltrated Lymphocytes
TLR	Toll Like Receptor

TMZ	temozolomide
TNF	Tumor Necrosis Factor
TPR	Tyrosinase-Related Protein
Treg	regulatory T cell
TSA	Tumor Specific Antigens
US	United States
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WT	Wilm's Tumor
Y	Yttrium

ABSTRACT

Glioblastoma (GBM) is the most frequent and aggressive brain tumor in adults. The patients' median survival is only 14.6 months despite multimodal treatment, including surgery and concomitant radiation and chemotherapy. Thus, there is a stark need for the development of new, potent therapeutic strategies and targets. For a decade our group investigated the implication of the cell surface neuroglial-2 proteoglycan, NG2/*CSPG4*, in the development and growth of glioma. These studies revealed that NG2/*CSPG4* is critical in multiple mechanisms that favor tumor survival and expansion. Indeed, GBM cells expressing NG2/*CSPG4* were characterized to have a higher proliferation rate and migratory capacity *in vitro*. In addition, *in vivo* implantation of GBM cell lines expressing this proteoglycan resulted in a bigger tumor mass, with a higher neovascularization compared to its negative counterpart. Furthermore, these NG2/*CSPG4* positive neoplastic cells were more resistant to chemotherapy and radiotherapy *in vitro* and *in vivo*. We further demonstrated that 50 % of GBM patients expressed NG2/*CSPG4*, and that this expression independently correlated with a poorer survival. Taken together, this proteoglycan might be an amenable target for immunotherapy. On the other hand, our research team has high expertise on natural killer (NK) cell biology. We observed that despite the promising results described for NK cell based immunotherapy to treat hematological malignancies, there is sparse data on their use to treat GBM.

The main goal of this thesis was the study and the validation of a novel combination treatment using mAb9.2.27 targeted against NG2/*CSPG4* and activated NK cells to treat GBM in rat orthotopic xenograft models. Thus, we first investigated the presence and phenotype of NK cells in the healthy brain. We further characterized the immunological status of GBM patients, both within the tumor microenvironment and their peripheral blood. Then we designed a novel purification method to obtain highly pure "untouched" NK cells from rat. This last method was developed to be able to finally investigate the efficacy of the combined NK+mAb9.2.27 treatment in GBM -bearing rats. All this work led to 4 original publications.

The first publication of this thesis revealed that NK cells could be found in the brain of naïve mice as well as in the cerebrospinal fluid obtained from non-pathological brain of patients. Phenotypically, brain NK cells displayed immature $CD11b^{low}CD27^{+}$ phenotypes in mice and they were mostly $CD56^{bright}$ in patients. In this publication we discussed these new findings in relation to the existing literature on systemic and brain NK cells in the context of central nervous system (CNS) disorders, such as brain tumors, infections, neurodegenerative diseases and mental disorders. We observed that the knowledge on brain NK cells is limited in the context of CNS disorders, though more information about the presence and role of NK cells in the peripheral blood of patients with such disorders is readily available. Nevertheless, this work distinguished that NK cells could be either implicated in neuroprotection or neurodegeneration. This review highlights the potential of a better comprehension of NK cells in brain pathogenesis that could further help to delineate new therapeutic targets for the treatment of CNS disorders.

In the second paper, we performed the characterization of the immunological status of GBM patients, in order to delineate the interplay between the immune system and tumor that can favor tumor growth. Immunohistochemistry staining on 65 GBM biopsies revealed that the patients' survival correlated with their infiltration by $CD3^{+}$ cells as well as $CD8^{+}$ cells. We further observed that following tumor infiltration the T lymphocytes as well as antigen presenting cells down modulated their activation molecules and up regulated their inhibitory molecules. Furthermore, we characterized for the first time the presence of infiltrated regulatory T cells with

$CD8^+CD28^-Foxp3^+$ phenotype that may further propagate the anti-inflammatory environment created by the tumor. Despite these findings, further analyses indicated potential for immunomodulatory therapies for GBM management.

The third paper of this thesis detailed a novel protocol to obtain highly pure "untouched" rat NK cells, based on magnetic-bead purification method. As currently no commercial kits to get untouched rat NK cells exist, many research teams used positive selection to purify them. Our method permits the separation of NK cells from athymic nude, Lewis and Fisher rat strains with high purity, ease, and cost affectivity and without bias of method-related activation artifacts. Indeed, we observed that positive selection of NK cells modified their proliferative and functional capacities. We conclude that for future fundamental studies, negative purification should be preferred to limit activation bias induced by separation methods.

This method was further applied to purify NK cells in order to investigate their efficacy as monotherapy or in combination with mAb9.2.27 against NG2/*CSPG4* as a new strategy to treat GBM. This NK+mAb9.2.27 treatment was evaluated in several orthotropic models of rats bearing GBM tumors. We observed that this combined therapy prolonged animal survival compared to monotherapy controls. This was associated with a diminution of tumor mass, associated with increased cellular apoptosis and diminished proliferation in the tumor bed. The levels of pro-inflammatory IFN-γ and TNF-α, were increased in the brain of treated animals, while in contrast anti-inflammatory IL-10 molecules were reduced. We further identified tumor recruited pro-inflammatory macrophages as the mechanism that mediated the anti-tumor immune responses as their depletion by clodronate abolished tumor destruction.

All together, we propose that targeting the anti-inflammatory interplay promoting GBM progression with combined NK cells and mAb9.2.27 against NG2/*CSPG4* could be amenable to treat GBM patients, by reversing the anti-inflammatory tumor microenvironment to a pro-inflammatory one in order to reveal GBM to the immune system.

1. LIST OF ARTICLES

This thesis is based on the following PAPERS that are referred to in the text by roman numerals.

PAPER I

Natural Killer Cells in Central Nervous System Disorders.

Poli A, Kmiecik J, Domingues O, Hentges F, Bléry M, Chekenya M, Boucraut J and Zimmer J.

J Immunol. 2013 Jun 1;190(11):5355-62

PAPER II

Elevated CD3+ and CD8+ tumor–infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor and peripheral microenvironment.

Kmiecik J, **Poli A**, Brons NHC, Waha A, Eide G, Enger PO, Zimmer J and Chekenya M.

J Neuroimmunol. 2013 Nov 15;264(1-2):71-83

PAPER III

Novel method for isolating untouched rat natural killer cells with higher purity compared with positive selection and fluorescence-activated cell sorting.

Poli A, Brons NH, Ammerlaan W, Michel T, Hentges F, Chekenya M, Zimmer J.

Immunology. 2010 Nov;131(3):386-94.

PAPER IV

Targeting glioblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival.

Poli A, Wang J, Domingues O, Rygh CB, Yan T, Thorsen F, Planagumà J, McCormack E, Hentges F, Pedersen PH, Zimmer J, Enger PO and Chekenya M.

Oncotarget. 2013 Sep;4(9):1527-46

2. INTRODUCTION

Cancer represents a leading cause of mortality in developed countries and the second cause in developing countries [15]. About 12.7 million new cancer cases and 7.6 million cancer deaths were reported in 2008 (GLOBOCAN 2008). Breast cancer in females and lung cancer in males are the leading cause of cancer related deaths. The global burden of cancer continues to grow, due to population aging, worldwide adoption of cancer-causing behaviors, in particular cigarette smoking, and physical inactivity. While the incidence of cancer in developed countries represents twice that of the developing countries, the overall mortality is equivalent [15], thus cancer represents a major health and economic issue worldwide.

Brain cancer accounts for 1.7 % of all new cases of cancer reported worldwide [16]. Although this is a low incidence, it represents a frequent form of cancer related deaths with about 3.5 % of the total cancer death rate [17]. Central nervous system (CNS) tumors consist of a heterogeneous group of benign and malignant forms. This work was performed on glioblastoma (GBM), which represents the most frequent form of malignant brain tumors.

Brain tumors are distinct from other solid tumors due to the cells of origin and the unique microenvironment of the CNS. The brain tumor microenvironment consists of specific cell types such as microglia, glial cells (astrocytes, oligodendrocytes and ependymal cells), neurons and vascular cells (endothelial cells and pericytes). Furthermore, brain tumors are infiltrated by different types of hematopoietic derived-cells [12]. During the last decade, new knowledge on GBM biology has led to the development of novel cellular and molecular targeted therapies aimed at improving current treatment.

The work presented herein consists of the development and validation of a novel combination immunotherapy using natural killer (NK) cells and mAb9.2.27 against NG2/*CSPG4* to treat GBM. We first characterized GBM biopsies of patients from a Norwegian cohort for their immunological status: i.e in the tumor microenvironment and systemically in peripheral blood. We also developed tools and used state-of-the art animal models to validate this new treatment strategy.

This introduction will first introduce GBM classification, diagnosis and treatment in the context of other brain tumors, and review the cross talk of GBM cells with the immunological environment. Then, the major tumor specific antigens (TSA) in GBM and their functions will be briefly reviewed focusing on NG2/CSPG4, the molecular target of interest. An overview of immunotherapies existing for GBM in preclinical and clinical trials will be provided. This introduction will conclude with the description of NK cell functions and their potential as potent effectors in immunotherapy.

2.1. Classification, incidence and prognosis of GBM

2.1.1. Incidence

In the United States during the period from 2005 to 2009 the incidence rate of gliomas was 6.03 newly-diagnosed cases per 100,000 people per year [18]. GBM represents more than 50 % of all primary brain tumors, with an incidence rate of 3.19 cases per 100,000 per year [18]. Males are more susceptible than females, with 3.98 compared to 2.53 newly diagnosed cases per 100,000 per year, respectively [18]. The incidence of GBM increases in the elderly, with highest

incidence in the age group 75 to 84 years. GBMs are rare in children (019 years olds) with approximately an incidence of 3 % of all CNS tumors [18].

2.1.2. Diagnosis

At the time of the diagnosis GBM patients generally present symptoms of neurological deficits such as cognitive impairment, headache, vomiting and/or seizures. These neurological symptoms are related to the tumor location and elevated intracranial pressure. Gliomas can occur in all lobes of the brain. After physical examination, neuro-imaging techniques are used for accurate diagnosis and patient follow-up. The imaging techniques include magnetic resonance imaging (MRI) with T1-weighted spin-echo (SE) sequence with and without gadolinium contrast agent infusion, T2 fluid-attenuated inversion recovery (FLAIR). Brain tumors are generally revealed by a poorly circumscribed low signal area on T1-weighted MRI, without contrast, and hypersignal on T2 weighted sequences or FLAIR. By MRI, GBMs often appear as ring-enhancing lesions, however as this image could resemble other disease entities, such as metastasis or abscess [19], the definitive diagnosis requires a stereotactic needle biopsy or a craniotomy with tumor resection and further pathologic confirmation. In addition to the current methods, multimodal MRI is more and more employed to provide detailed information about the tumor cellularity, metabolism and angiogenesis [20]. However, positron emission tomography (PET) with radiolabelled tracers like ^{18}Fluorodeoxyglucose (^{18}FDG) is preferred in order to detect tumor recurrence and distinguish it from radiation necrosis or pseudo-progression in some clinical centers [21].

2.1.3. Classification and grading

Malignant brain tumors consist of heterogeneous cancers including brain metastasis that arise upon dissemination from a primary neoplasm outside the CNS and primary brain tumor that originates from the endogenous glial cells. Gliomas are characterized by a multitude of histological features, related to the morphological similarities of the neoplastic cell with the normal, putative glial cell of origin. Gliomas are classified, by the World Health Organization (WHO), into 3 basic categories: (i) astrocytic, (ii) oligodendroglial and (iii) ependymal, although mixed glioma variants also exist [22]. In addition, each tumor category is further sub-divided into several grades of malignancy, ranging from lowest grade I to highest grade IV, based on histological criteria, nuclear atypia, mitotic activity, endothelial proliferation and or the presence of necrosis [22]. Grading is thus related to the level of malignancy delineated through the presence of these histological characteristics and is considered as an important prognosis factor. Thus, high grades, III and IV, are characterized by the presence of at least two criteria, usually nuclear atypia and mitotic activity (grade III), whereas the diagnosis of GBM (grade IV) requires additionally endothelial proliferations and/or pseudopalisading necrosis. These features are associated with a more aggressive clinical course leading to a considerably shorter patient survival time compared to tumors of lower grades (I and II) [22-24].

2.2. Biological behavior of GBM

2.2.1. Proliferation

GBMs are characterized, compared to lower grade gliomas, by an abundant cell proliferation, represented by a numerous of mitotic figures. Ki-67 is a nuclear protein expressed during the G1, S, G2 and M phases of the cell cycle, and has been widely used as a marker for cell proliferation in various tumor types [25, 26]. Immunohistochemical staining of Ki-67 is classically used to characterize the GBM growth factor rate, by Ki-67 labeling indices (LI). While its level of expression may correlate with survival [27], its use as a diagnostic marker in GBMs is still controversial [28].

2.2.2. Invasion

Diffuse infiltrative growth of cancer cells in the brain parenchyma is a unique feature of gliomas. This invasive nature was characterized early in the 40's when it was shown that the neoplastic cells show extensive migration into the brain parenchyma also invading the contralateral hemispheres [29]. The invading cells follow blood vessels through perivascular spaces, are disseminated passively through the cerebrospinal fluid (CSF) and migrate along white matter tracts as for instance the corpus callosum [29] to give rise to *so-called* "butterfly" GBM and multifocal gliomas [30]. This invasive nature is the major limitation for a curative surgical resection and is still the major cause of recurrence. Furthermore, the invading cells may show a reduce transcription of pro-apoptotic and proliferation genes, -decreasing their susceptibility to cytotoxic agents [31, 32].

2.2.3. Neovascularisation

The growth of GBMs is dependent on the availability of metabolites and oxygen [33]. Paradoxically, GBMs are characterized by hypoxic and necrotic palisades, caused in part by vessel regression [34] and concomitant increase of cellular proliferation [35]. This hypoxic environment leads to activation of the transcriptional complex hypoxia inducible factor-1 alpha (HIF-1α) followed by the up-regulation of vascular endothelial growth factor (VEGF) secretion and the subsequent formation of new blood vessels [33]. These new vessels are often arranged in glomeruloid formation.

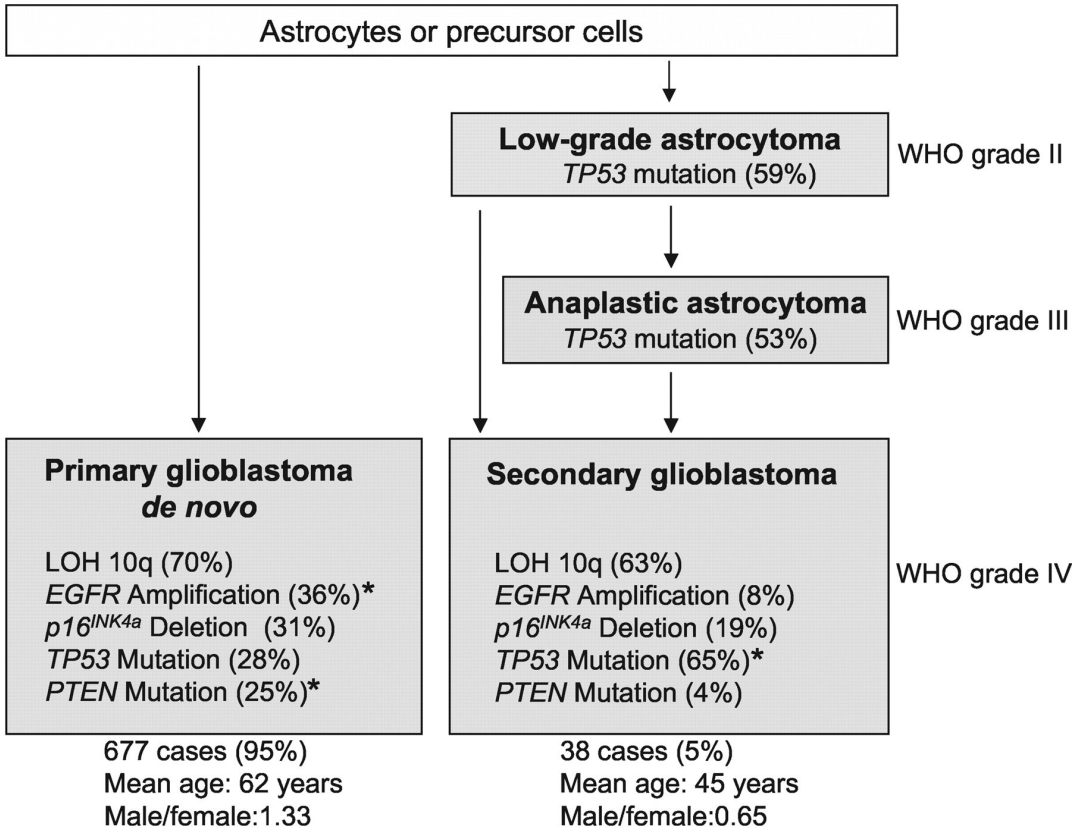
At least five mechanisms of neovascularization in brain tumors are proposed, including vascular cooption, angiogenesis, vasculogenesis, vascular mimicry, and glioblastoma-endothelial cell transdifferentiation. These mechanisms co-exist and are interlinked within the same lesion and have been recently extensively reviewed by Hardee et al [36]. Briefly, diffusely invading glioma cells initially grow around existing brain vessels by cooption. When the cancer mass has increased significantly, it leads to subsequent hypoxia-induced VEGF expression, where new vessel sprouting becomes critical for the growth of the tumor. Angiogenesis represents sprouting of pre-existing vessels, which is regulated by proteases that disrupt the basement membrane allowing endothelial cells to escape from the parent vessel walls. Then under the influence of angiogenic growth factors such as VEGF or basic fibroblast growth factor (bFGF), endothelial cell proliferation into the surrounding matrix forms the solid sprouts connecting neighbouring vessels. The third mechanism of neovascularization is vasculogenesis, where new endothelial cells are generated from the recruitment of bone marrow derived endothelial progenitor cells (EPC). The involvement of vasculogenesis in new blood vessel formation in glioma is still controversial as different studies show contradictory results [37-39]. Based on current information, the tumor's incorporation of EPC and their differentiation into endothelial cells to

promote tumor growth may depend on the tumor grade. Also, studies have shown possible involvement of vascular mimicry in GBM neovascularization [40, 41]. This process is characterized by the capacity of neoplastic cells to form functional and perfused vessel-like networks. The last mechanism of neovascularization may also occur independent of endothelial cells and represents a *trans*-differentiation of GBM cells into endothelial cells forming vascular channels. This recently described mechanism is induced by hypoxia [42], and can also result from the differentiation of glioma stem like cells into endothelial cells [43].

These newly formed blood vessels are necessary for tumor growth by supplying nutrients and oxygen, and may also represent a route for immune cell infiltration. Furthermore, the spread of tumor cells has been shown to follow a route traced by blood vessels. In this context, metastatic cancer cells have been shown to migrate between endothelial cell and astrocyte foot processes [44]. In addition, this interaction of cancer cells with the vascular wall may activate their invasive phenotypes via fibronectin engagement, resulting in subsequent activation of downstream signalling [45].

2.3. Molecular Genetics of GBM

Figure 1: Genetic pathways leading to primary and secondary GBM.

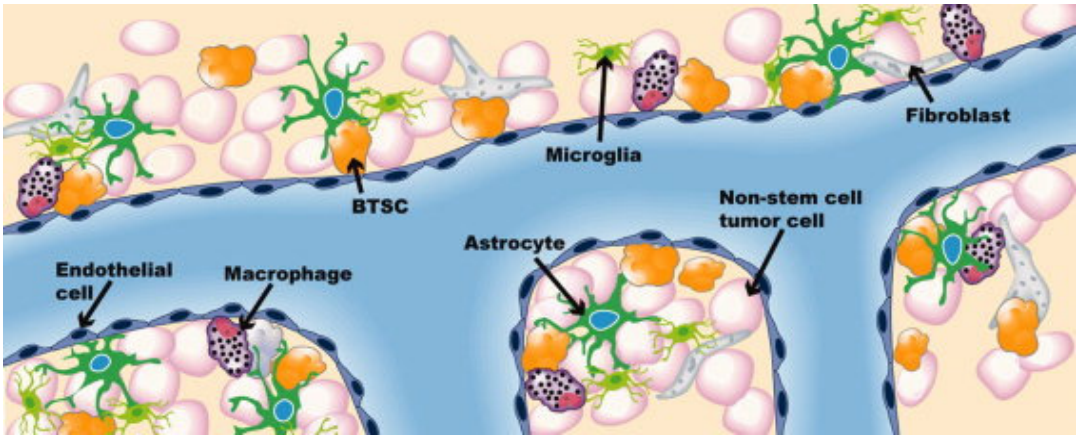


Molecular pathways leading to primary (*de novo*) GBM (left) and secondary GBM (right). *Genetic alterations that differ significantly in frequency between primary and secondary glioblastomas. Reproduced with permission of American Association for Cancer Research, published by [4].

In the 1940ties, Scherer distinguished primary (*de novo*) from secondary GBM [29]. It is now accepted that primary GBM is the most frequent form representing more than 90 % of the GBM, mainly affecting elderly patients [46]. This form is characterized clinically following focal neurological symptoms, and by showing contrast enhancing lesion on MRI without showing evidence of less malignant pre-existing lesions. Secondary GBMs are less frequent and affect younger individuals. They usually develop more slowly from pre-existing anaplastic astrocytoma or low grade astrocytoma [4, 46]. Later, Watanabe et al. confirmed the existence of these 2 distinct forms of GBMs on the basis of their differences in genetic alterations [47] and showed evidence for their development involving distinct genetic pathways [48]. For instance, there is a high incidence of TP53 mutations and loss of heterozygosity at 19q in secondary GBMs, while this is not common in primary GBMs. Inversely, primary GBM shows overexpression of *EGFR*, *PTEN* mutation and complete loss of chromosome 10, however these genetic modifications are rare in the secondary GBMs [4, 48-50]. Although informative, these genetic alterations did not permit an explicit distinction between the two subtypes until the discovery of the mutation in the active sites of isocitrate dehydrogenase 1 and 2 (IDH1/2) in secondary GBMs [51, 52]. Gene expression profiling of tumor biopsies further confirmed that primary and secondary GBMs represent separate genetic diseases even if some genetic alterations are common to both types [53, 54]. Furthermore, high-resolution copy number analyses using oligonucleotide-based array comparative genomic hybridization reveal the existence of two subgroups of secondary GBMs with significant difference in clinical outcomes [54].

2.4. The GBM microenvironment

Figure 2: Cellular constituents of the tumor microenvironment.



The brain tumor microenvironment consists of various cell types that make distinct contributions to tumor progression and invasion. These cells include but are not limited to astrocytes, macrophages, pericytes, fibroblasts, and endothelial cells. BTSC= Brain Tumor Stem Cells or Glioma Stem cells = GSC. Reproduced with permission of John Wiley & Sons Inc, published in [12].

The tumor microenvironment has come in vogue due to the recent association between the malignant progression and the roles of different stromal cells constituting the lesion [12]. This revived area of research might lead to the discovery of suitable targets for anti-cancer therapies as well as powerful prognostic markers [55, 56].

Due to the infiltrative growth, GBM cells cannot be clearly dissociated from their stromal cell microenvironment (Figure 2). Furthermore, an increasing number of studies describe the role of stromal cells in GBM proliferation, infiltration and recurrence [12]. This section will review the current knowledge gained related to the major cellular subtypes constituting the GBM tumor microenvironment, such as brain endothelial cells, pericytes, immune cells, astrocytes and glioma stem cells (GSC).

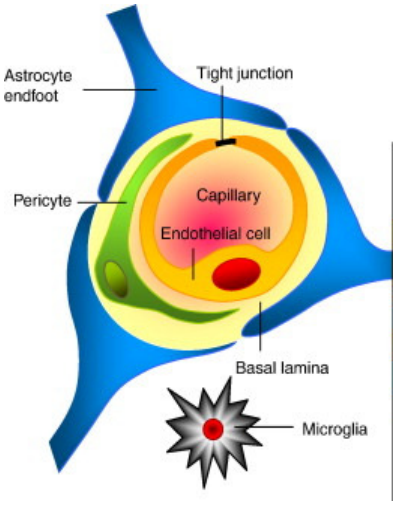
2.4.1. Endothelial cells and blood vessels

The brain is highly vascularized, mainly by capillary blood vessels that present unique structural and functional features compared the vasculature in tissues. The endothelial cells are interconnected by tight junctions, composed of lipid/protein-supra-structures [57], that restrict the passage of cells, hydrophobic compounds and large molecules (> 100 kDa) from the circulating blood to the brain parenchyma and interstitial fluids. A thin basal lamina, composed of laminin extracellular matrix and smooth muscle cells, supports the abluminal surface of the endothelium. Pericytes contact the basal lamina and astrocytes' end-feet cover the vascular structure and maintain the integrity of the *so-called* blood brain barrier (BBB) or neurovascular units (Figure 3) [14].

However, the BBB is variably disrupted in GBMs, with regional variations demonstrating leakiness, as visualized by immunohistochemistry staining of plasma proteins on patient biopsy tissues [58] as well as by heterogeneous dynamic contrast enhancement upon MR imaging [59]. Furthermore, Pronin et al. demonstrated that the degree of breakdown of the BBB corroborates with the volume of edema surrounding gliomas, as determined by gadolinium contrast enhancement [60]. Permeability leads to elevated intracranial pressure that is the major source of the focal symptoms of a brain tumor. The mechanisms leading to BBB permeability in glioma are not fully understood, as recently reviewed by Wolburg et al. [61]. However, the integrity of tight junctions is lost through the activation of matrix metalloproteases (MMPs) such as MMP-3 that degrade proteins, such as agrin and claudin-3 that are major components of thigh junctions. This results in a dissociation of pericytes and astrocytes from the vessel wall and an increased volume of the perivascular space. This increased permeability leads to an influx of systemic molecular compounds and cells that may promote tumor growth [62].

Pericytes are derived from mesenchymal stem cells (MSC) [63, 64], are essential for the maintenance of BBB integrity (Figure 3) and for the regulation of hemodynamic responses in the vasculature [65]. In addition, these cells modulate the CNS blood flow and help in the elimination of toxic cellular products [66]. Pericytes are characterized by their expression of platelet derived growth factor-beta (PDGF-β), α-smooth muscle actin (α-SMA), desmin and neuron glia 2/chondroitin sulphate proteoglycan 4 (NG2/*CSPG4*). These markers are not specific to pericytes and can be differentially expressed depending on their level of maturation.

Figure 3: Transversal view of the BBB.



The cerebral endothelial cells form tight junctions at their margins that limit passage of elements from the blood to the brain parenchyma. Pericytes are distributed discontinuously along the length of the cerebral capillaries. Pericytes and endothelial cells are enclosing that form the basal lamina. The foot processes from astrocytes form a complex network surrounding the capillaries and this close cell association is important in induction and maintenance of the barrier properties. Reproduced with permission from Elsevier, published in [14].

2.4.2. Pericytes

During blood vessel formation, MSC are recruited through paracrine PDGF-β secretion by endothelial cells that favors vascular tube development [67 131]. Indeed, pericytes have a major role in angiogenesis by organizing initiation, sprout connection and termination via expression of transforming growth factor-β (TGF-β), VEGF, and angiopoietin-1 and -2 [68]. Moreover, NG2/*CSPG4* expression on pericytes has been shown to be essential for their recruitment to vessel walls, their interaction with endothelial cells and their maturation both in normal brain and brain tumor [69-71]. NG2/*CSPG4* will be discussed more in detail later in this introduction, as it represents the salient molecular target of this work. Briefly, NG2/*CSPG4* was demonstrated to be essential for endothelial cell morphogenesis and angiogenesis via engagement of galectin-3 and α3β1-integrin [72]. The extinction of NG2/*CSPG4* transcript *in vivo* by interfering RNA or by mouse knockdown resulted in diminution of tumor mass and vasculature, as well as increased survival and chemoresistance of GBM bearing animals [70, 73, 74]. Clearly the recruitment of pericyte progenitor cells is essential for the survival of tumor endothelial cells [64] and the development of microvascular proliferating structures in GBM [75]. Other studies pointed to the role of pericytes in promotion of angiogenesis [76] and the elaboration of a tumor neovascular tree [63].

2.4.3. Astrocytes

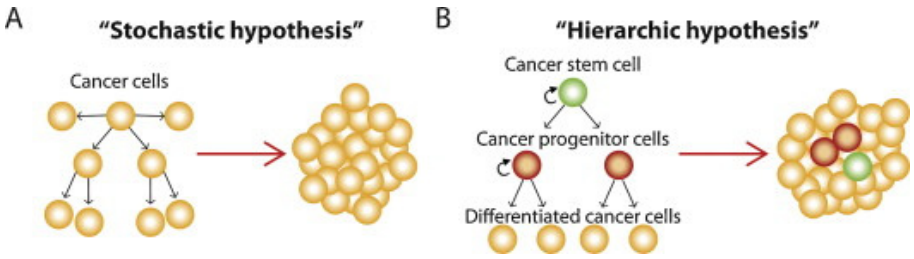
Astrocytes represent the most abundant glial cells of the brain found in close contact with neurons and with the BBB through their terminal end-feet processes (Figure 3). They have many functions including structural support and roles in synaptic transmission within CNS. They have additional roles to provide nutriments to neurons, such as lactate, the neurotransmitter glutamate and brain derived neurotrophic factor (BDNF) release [77].

Astrocytes present structural modifications following CNS injury, including GBM, a phenomenon called astrogliosis [78]. This is characterized by cellular hypertrophy and modifications of gene expression patterns, in particular, over-expression of glial fibrillary acidic protein (GFAP). The reactive, $GFAP^+$ astrocytes are often described in the vicinity of primary

brain tumors including GBM [79]. Furthermore, it was demonstrated that glioma can modulate the phenotype of astrocytes, such as their motility [80] and proliferation [81] *in vitro*. On the other hand, astrocytes were implicated in the invasiveness of glioma via activation of pro-MMP2 [82], a metalloproteinase implicated in glioma invasion [62]. Astrocytic activity was correlated with tumor grade in a model of PDGF induced glioma [83]. These last examples highlight that glioma modulate neighbouring astrocytes for their invasiveness; but there is no experimental evidence to elucidate if astrocytes could participate in GBM proliferation *in vivo*. Nonetheless, astrocytes were shown to secrete stromal cell-derived factor-1 (SDF-1) [84, 85], as GBM proliferation was aberrantly induced by SDF-1/CXCR-4 signaling axis [86]. Therefore, we can speculate that astrocytes may activate tumor growth. Finally, in the same way, factors secreted by astrocytes, such as TGF-α, CXCL-12, S1P and glial cell-derived neurotrophic factor (GDNF), may have the capacity to modulate the invasiveness of GBM [87]. Furthermore, astrocytes were shown to up-regulate their expression of MHC class II molecules and to present antigen to T cells *in vitro* [88]. However, in the context of glioma, these capacities were shown to be suppressed [89].

2.4.4. Glioma stem cells (GSC)

Figure 4: **Schematic representation of the stochastic and hierarchical models of tumor initiation and progression.**



(A) Stochastic model: in this model all neoplastic cells present equal proliferative ability. Genetic mutations and signals from the microenvironment apply evolutionary pressures able to select for tumor cells with different phenotypes, aggressiveness and tumourigenic potential. **(B)** Hierarchical model: according to this model only the CSCs are able to self-renew and to originate Cancer Progenitor Cells (CPCs). CPCs display higher proliferation and may give rise to differentiated cancer cells. In this context the resulting tumor is composed by a hierarchy of cells gradually differentiating from a CSC. Only the CSCs are able to re-initiate tumor progression. Reproduced with permission of Elsevier, published in [10].

Recently the discovery of cancer stem cells (CSC) led to a new vision on tumor development and progression, as these cells are suspected to play a role in tumor initiation and maintenance in a multitude of tumor types, including glioma [90-92]. Two models of tumor formation co-exist (Figure 4). The classical "stochastic model" states that any individual cell in the pre-tumoral lesion may function as tumor initiating cell and that further, all neoplastic cells have equal tumorigenic potential. Thereafter, the tumor results in a mass of hyper proliferative cells that gains several mutations in relation with the microenvironment (Figure 4A). On the other hand, recent experimental evidence supports the "hierarchic hypothesis" stating that the tumor mass is initiated by a unique CSC population, presenting high proliferative rate, the ability to self-renew and the possibility to give rise to differentiated cancer cells [93](Figure 4B).

GSC share common features with CSC such as the ability to self-renew and their capacity to differentiate into heterogeneous tumor cell types. Furthermore, their elevated resistance to chemo- and radiotherapy could explain why current therapies fail to cure patients and lead invariably to post-surgery recurrence [94, 95]. The discovery of GSCs opens a new therapeutic focus, where targeting GSC, instead of the tumor bulk may disable remaining tumor cells to sustain further growth. There is a distinct phenotype related to GSCs, for example CD133 expression, and molecular profiles, when compared to the non-neoplastic stem cells [96, 97]. However, the existence of GSC is still controversial, as many uncertainties remain regarding theoretical, technical, and interpretational aspects of the data supporting it [98, 99].

Studies reported that CD133 expression levels became significantly higher as the glioma grade advanced [100, 101]. However, this concept of GSC becomes controversial in regard of conflicting results. Indeed, the relation of tumor emergence and maintenance by GSC should be reflected by a correlation between the amount of GSC cells in patient biopsy and their survival outcomes. While several studies observed that the stem cell marker CD133 affects clinical outcome in glioma [100, 101] others did not find any correlation between frequency of $CD133^+$ cells and patient survival [102]. It should be noted that the use of CD133 as a marker of GSC is limited since $CD133^-$ cells have been shown to have CSC characteristics and can give rise to tumors [99, 103, 104]. Furthermore, the choice of the mAb clone for the characterization of $CD133^+$ could reflect some inconsistencies seen between studies as it may lead to different results [105]. Nonetheless, it seems that patients with the highest level of $CD133^+$ cells in their biopsy also presented poorer response to chemotherapy [103, 106, 107]. Moreover, it appears that $CD133^+$ cells presented differential molecular profiles than $CD133^-$ ones, while $CD133^+$ neoplasms presented a profile in favor of blood vessel formation, angiogenesis, permeability and invasiveness implicated in tumor progression [103, 106].

GCS are found in specialized microenvironments, such as hypoxic and perivascular niches. It has been demonstrated that hypoxia promotes GSC function but also induced phenotypic modifications of cancer cells towards GSC phenotypes [108]. In addition, GSC have been shown to be closely associated with endothelial cells within the perivascular niche [109]. Endothelial cells secrete important factors, such as VEGF and nitric oxide, for the maintenance of the GSC pool, and have been observed to influence the transformation of neural stem cells (NSC) into GSC, further accelerating their tumorigenic capacities [109, 110].

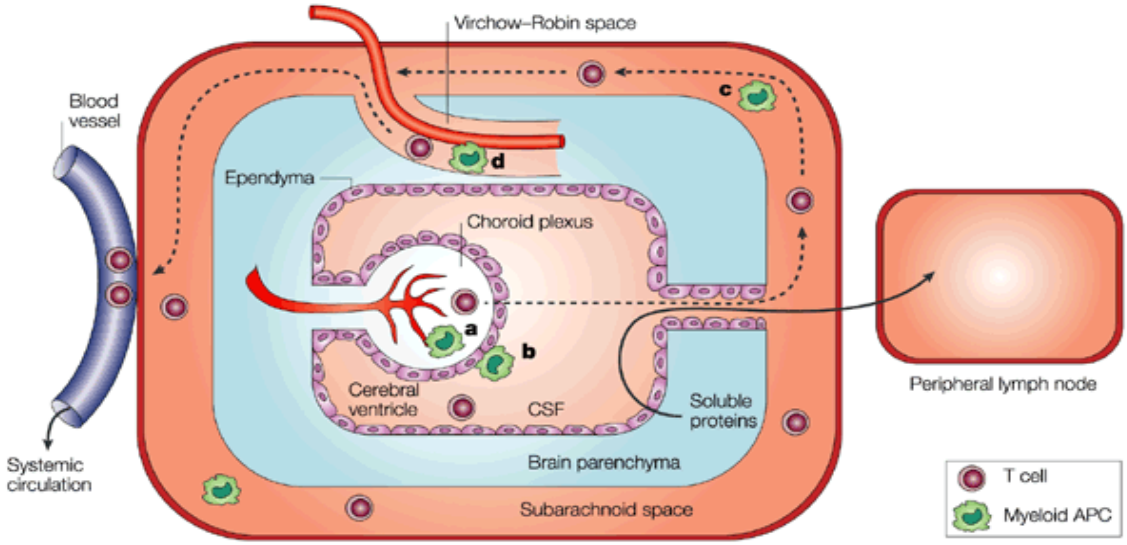
2.4.5. Immune cells

2.4.5.1. Actors of CNS immunity

The prevailing view is that immune cells undertake immune surveillance of the CNS. The brain immune system includes the presence of unique, resident innate immune cells, the microglia, in brain parenchyma. In addition, CNS-associated macrophages were also characterized in brain choroid plexus (Kolmer cells), in ventricles, in the meninges and in perivascular space (Virchow Robin-space), (Figure 5 a,b,c and d respectively). Brain antigen present in the CSF can trigger antigenic stimulation of T cells from the cervical lymph nodes via lymphatic channels to peripheral lymph nodes (Figure 5). In addition, the brain has an adaptive immune system that is represented by the circulation of memory T cells [111]. These cells are trafficking from the systemic blood to the CSF through the choroid plexus and return back to

systemic circulation through arachnoidal villi (Figure 5) [7]. Recently, we identified a population of immature Natural killer (NK) cells in the normal CNS, however their implication in brain physiology and function remains elusive and requires further exploration (PAPER I).

Figure 5: Schematic representation of the mechanism of immune surveillance in the CNS.



The choroid plexus, cerebral ventricle, subarachnoid space, brain parenchyma, blood systemic circulation and a peripheral lymph node are represented in a cartoon. The soluble proteins could stimulate T cells from peripheral lymph nodes by their transport through lymphatic channels. As indicated by dotted arrows, memory T cells can migrate from blood to the subarachnoid space and returning back to the peripheral blood. A mixed type of myeloid-lineage cells constitute the CNS including: choroid-plexus macrophages (a), epiplexus cells (b), meningeal macrophages (c) and perivascular cells of the Virchow–Robin spaces (d). Reproduced with permission of Nature Publishing Group, published in [7].

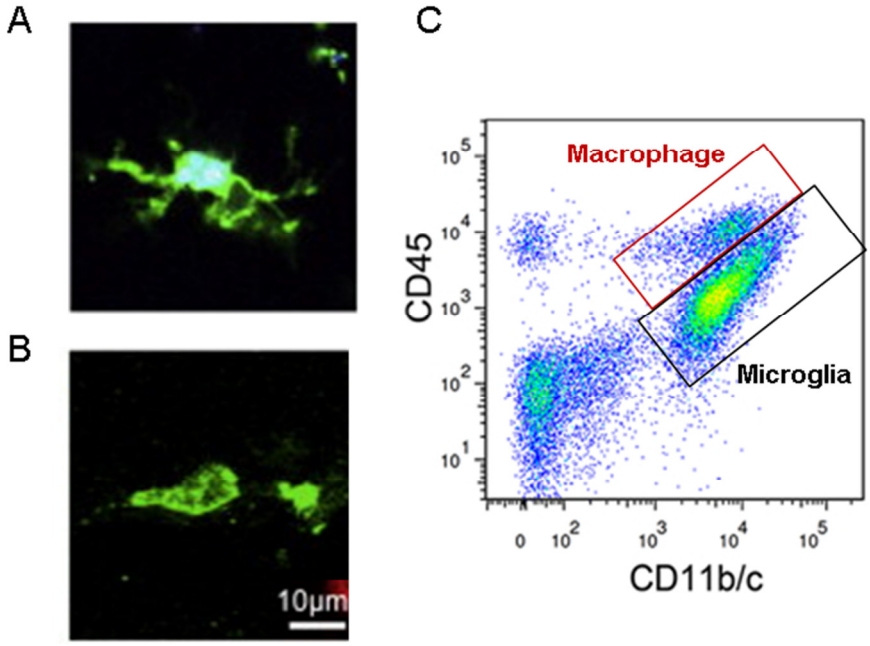
2.4.5.2. Microglia in healthy CNS

Microglia cells are considered specialized resident macrophages of the brain. These cells derive from extra-medullary sources of hematopoiesis, including the yolk sac. Thus, CNS microglia progenitors arise from a common myeloid progenitor to macrophages and invade the brain parenchyma during embryonic and fetal development [112]. Nonetheless, some authors postulate that a second wave of microglia progenitors may arise before birth and during the early postnatal period from bone-morrow derived monocytic cells [113]. While at steady states their replacement is slow and occurring from a non-bone-marrow source, by self-renewal of $CD34^{+}$ microglia [114, 115], during CNS disorders their number can rapidly increase, with a focal accumulation of activated microglia, by a process called microgliosis [116, 117]. This accumulation during brain pathologies is mainly dependent on self-renewal, but could also arise by differentiation of bone-marrow derived monocytes, as reported in mouse models of Alzheimer disease [118], meningitis [119] or traumatic brain injury [120].

Microglia are localized in the brain parenchyma in close contact with oligodendrocytes, neurons and astrocytes. Another population termed juxta-vascular microglia was characterized in interaction with the basal lamina of CNS blood vessels at the BBB [121]. Microglia perform several functions. During brain development they undergo phagocytosis of necrotic and

apoptotic cells to help in the elaboration of neuronal networks. In addition, in healthy brain they participate in the development of synaptic plasticity. Furthermore, in pathological CNS they can be either neurotoxic or neuroprotective, by release of cytotoxic factors such as reactive oxygen species or neurotrophic factors, respectively [122]. They can also perform phagocytosis and antigen presentation [123]. Microglia show high morphological plasticity in relation to their microenvironment [124]. In normal contexture, these cells have highly ramified morphology (Figure 6A), and present low expression of MHC class I and II molecules, CD40 and CD86 co-stimulatory molecules [113]. They permanently sample their microenvironment and after activation they can undergo several phenotypic changes characterized by up-regulation of MHC class I and II molecules, CD40, CD80 and CD86 expression, as well as rapid secretion of a large array of molecules (such as TNF-α, IL1-β and myeloperoxydase) that favor brain defense [113, 125, 126]. Upon activation, these cells attain a more rounded morphology and are thus denoted reactive amoeboid phagocytic microglia [123] (Figure 6B).

Figure 6: Microglia morphology and phenotype in rat following drug activation and tumor development.



(A) Resting ramified microglia and **(B)** activated amoeboid microglia following drug activation in retina of rat. Reproduced with permission of Elsevier (Figure modified from [1]. **(C)** Phenotype of macrophage and microglia from brain lysate of a tumor bearing rat, from PAPER IV.

2.4.5.3. CNS-associated macrophages and dendritic cells

In addition to microglia, the brain harbors CNS-associated macrophages with heterogeneity in their phenotypes, functions, turnover and localization. These *so-called* brain myeloid cells essentially include perivascular macrophages, meningeal macrophages, choroid plexus macrophages and dendritic cells (DC).

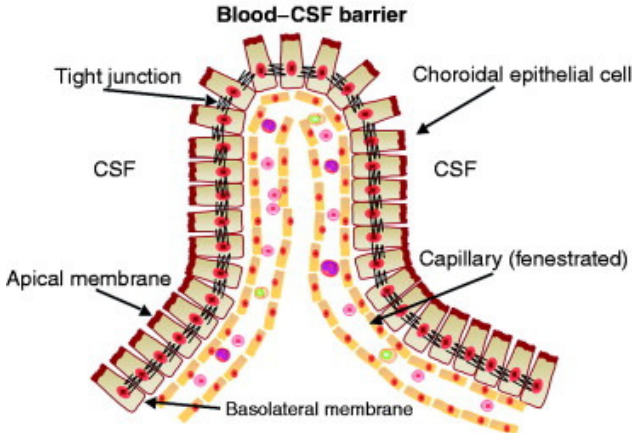
Phenotypically, microglia and CNS-associated macrophages are highly similar, making their distinction complicated by simple immunohistochemistry. However, by flow cytometry

they are both positive for CD11b/c but express differential levels of CD45, CD45 expression being typically higher on macrophages, CD11b/c^{+}CD45^{bright}, compared to microglia, CD11b/c^{+}CD45^{dim} (Figure 6C)[127].

Perivascular macrophages are found in the Virchow-Robin space, which is the small space filled with CSF/interstitial fluid around invading vessels in the brain parenchyma (Figure 5). These perivascular and meningeal macrophages have a unique phenotype compared to other CNS-associated macrophages as they are positive for CD68 (ED1) and CD163 (ED2). These cells are derived from specific subpopulations of CD163^{+} monocytes and stem cells from the periphery [128, 129]. These macrophages have a protective role during infection [130, 131] and could be implicated in tissue remodeling after brain injury [132].

Choroid plexus is the cuboidal, ciliated epithelial layer localized in the cerebral ventricles responsible for CSF synthesis. This structure also constitutes the blood CSF barrier (BCSFB), which is more permissive for the entry of blood borne elements compared to BBB, as it has fenestrated and permeable capillaries (Figure 7) [133].

Figure 7: Structure of the brain cerebral spinal fluid barrier.



Representation of polarized choroidal epithelial cells that are connected by tight junctions and vascularized within a fenestrated capillary bed. Reproduced with permission of Elsevier, published in [2].

The inner part of the choroid plexus includes macrophages, *so-called* choroid plexus macrophages and a subpopulation of DCs. In contact to the CSF, there are Kolmer cells, residing at the outer surface of the choroid plexus. These cells are probably reconstituted by blood elements and by self-renewal, and are considered as first line of defense of the brain, due to their cytotoxic and antigen presenting functions [134-136]. Furthermore, Nataf et al. proposed that the stroma of choroid plexus contains myeloid progenitors that may serve as a reservoir for brain-associated macrophages and DC [137].

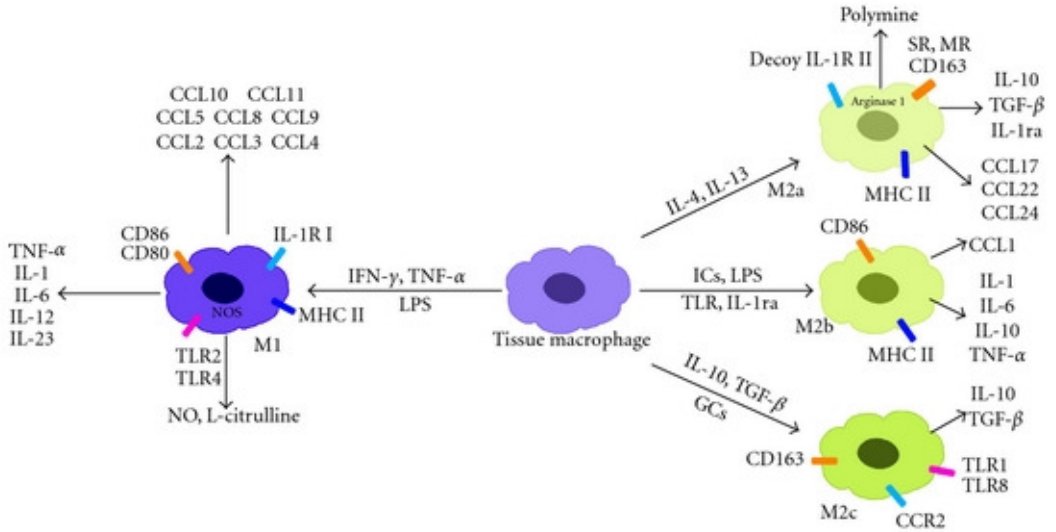
2.4.5.4. Glioma-associated macrophage/microglia

2.4.5.4.1. Polarization of macrophages according to *the microenvironment*

In pathological conditions, including tumors, macrophages have been shown to exhibit several polarizations in relation to their direct microenvironment, as has been described for T helper-1 and T helper-2 polarization of T cells [138]. There are two main types of macrophage

polarizations, called M1 or pro-inflammatory macrophages and M2 or anti-inflammatory or alternative macrophages. These alternative macrophages have further been separated into three subgroups in relation to their differential phenotypes and cytokines secretion profiles: M2a, M2b and M2c (Figure 8).

Figure 8: Schematic representation of macrophage polarization according to the microenvironment cytokine profiles.



Th-1 cytokines, LPS, IFN-γ, or TNF-α were shown to differentiate macrophages into classical M1 macrophages. Alternatively polarized macrophages were further divided into M2a, M2b, and M2c macrophages under the influence of Th-2 cytokines. IL-4 and IL-13 manipulate macrophages to be M2a macrophages, while M2b macrophages are activated by immune complexes, TLRs, or IL-1ra. And finally, M2c macrophages are polarized by IL-10. All of the phenotypes express a series of different cytokines, chemokines, and receptors. Obtained from [13](Open access article).

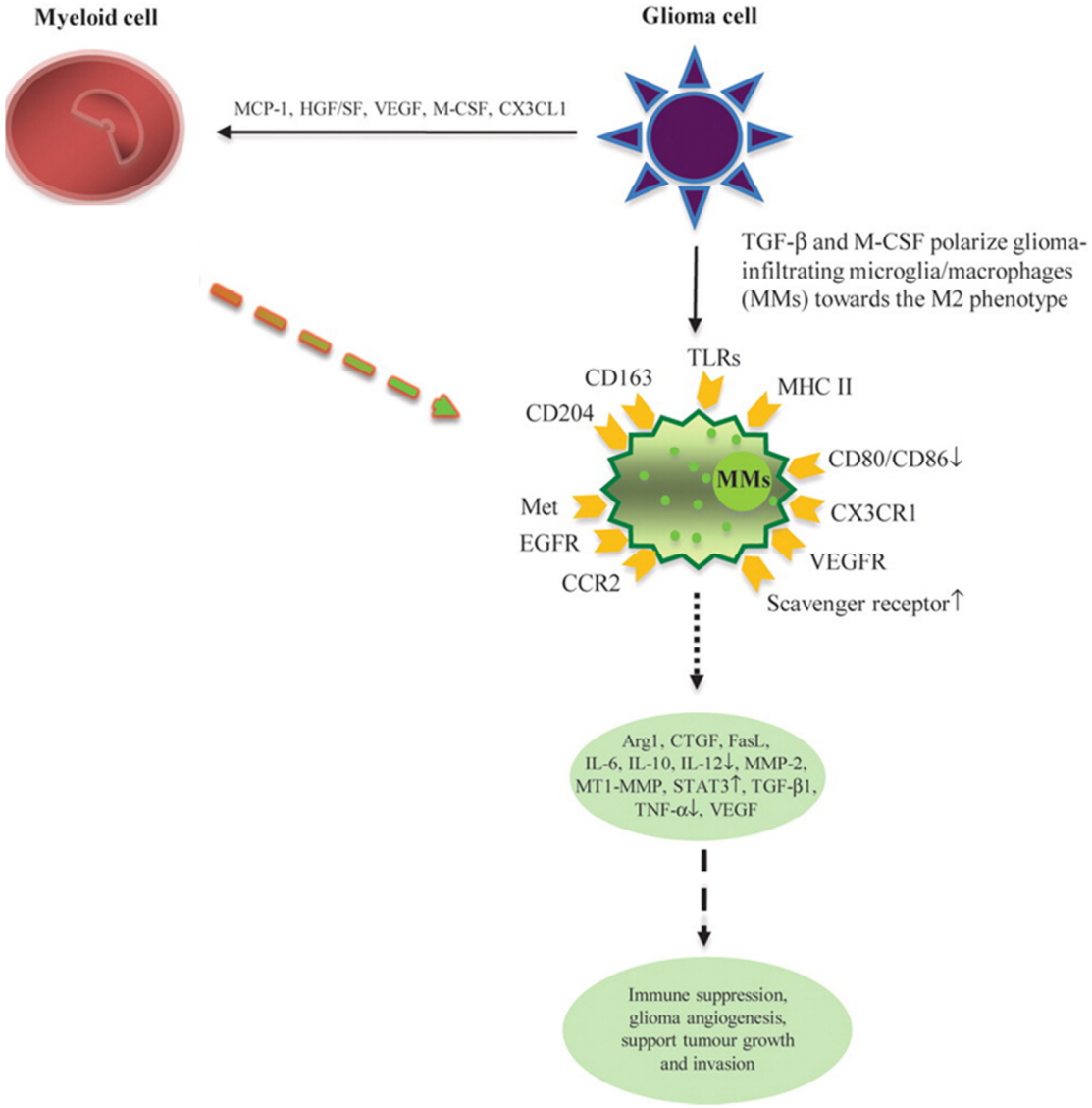
The role of M2 macrophages in the development of an immunosuppressive microenvironment that favors tumor growth is becoming more elucidated in a variety of tumor types, including gliomas [139, 140]. Indeed, there is a bidirectional cross talk between neoplastic cells and infiltrating macrophages leading to optimal conditions for tumor angiogenesis, growth, metastasis, and immunosuppression. Furthermore, it has also been demonstrated that tumor-associated macrophages interact with cancer stem cells, resulting in augmented tumorigenesis, metastatic potential, and drug resistance [141]. As a result, the presence of alternative macrophages in tumor biopsies is often of negative prognostic relevance [142, 143]. In contrast, the presence of pro-inflammatory M1 macrophages, which have direct and indirect cytotoxic capacity against cancer cells [144], has been shown to correlate with beneficial outcomes [145, 146]. Nonetheless, the polarization of tumor associated macrophages can sometimes not distinguish M1 or M2 specifically [147] as they could share common features to both of these populations [148, 149]. Moreover, the same tumor could present multiple macrophage polarizations depending on the inspected niche and their specific functions. For example, hypoxia promotes tumor-associated macrophages with angiogenic functions whereas oxygenated perivascular areas present macrophages that favor cancer metastasis [143, 150].

2.4.5.4.2. Polarization of macrophages/microglia in GBM

The implication of macrophage/microglia in GBM initiation [151] and progression has been intensively studied durin the last decade [11, 152]. Macrophage/microglia constitute up to 70 % of the GBM tumor mass [153] and are recruited by GBM-secreted chemokines, including MCP-1 [154], MCP-3 [155], M-CSF [156], as well as hepatocyte growth factor/scatter factor [157]. In addition, these glioma associated factors were shown to activate microglia proliferation [158]. The presence of macrophage/microglia has been correlated with size of the tumor mass [159], grades of malignancy [139] and their gene expression profiles have been associated with patients' survival [160, 161]. It appears that infiltrated macrophage/microglia presented altered phenotypes and functions. In fact, it becomes evident that GBM secreted molecules modulate macrophage/microglia that in turn favor tumor invasiveness and proliferation [11, 162](Illustrated in figure 9).

Glioma associated macrophage/microglia have an amoeboid activated phenotype, that is distinct from their resting morphology in the intact brain [159, 163]. Furthermore, glioma-associated macrophage/microglia lack expression of co-stimulatory CD40, CD80 and CD86 [164, 165] and MHC class II molecules expression, critical for T cell activation [89, 164, 166, 167]. Moreover, glioma infiltrated macrophage/microglia presented impaired secretory capacity of pro inflammatory cytokines interleukin-1β (IL-1β), IL-6 and TNF-α [168]. In addition, their cytotoxicity and phagocytosis capacities were reduced compared to microglia isolated from normal brain [168]. These altered phenotypes and function are mediated by glioma-secreted factors, IL-4, IL-6, IL-10, TGF-β, M-CSF and prostaglandin E2 (PGE2)[139, 169-172]. The glioma microenvironment favours M2 polarized macrophage/microglia with anti-inflammatory properties [139, 140, 173, 174]. Thus, the expression of CD163 and CD204, established M2 markers, correlated with histological grade of gliomas [139]. These cells were implicated in the tumor angiogenesis by their capacity to release VEGF [175]. They are also involved in local immunosuppression by their secretion of IL-10 [176] and TGF-β [173]. In addition, they express FAS-L that could lead to apoptosis of FAS expressing immune cells [177]. Moreover, tumor-associated macrophages/microglia enhance the invasion of glioma cells and GSC like cells via TGF-β1 signalling pathway, inducing MMP-9 that further degrades extracellular matrix [178-180]. In addition, M2 polarized macrophage/microglia express MT1-MMP in the context of glioma that in turn activates glioma-derived pro-MMP-2 and promotes tumor expansion [181 370]. They also facilitate glioma invasion by up-regulation of MMP-2 [182] and epidermal growth factor (EGF) [156]. This interaction between glioma and macrophage/microglia is well reviewed by Li et al (Figure 9) [11].

Figure 9: Schematic representation of the interplay between infiltrated myeloid cells and glioma.



Glioma secreted molecules have several functions, for example TGF-α and M-CSF can polarize glioma infiltrated macrophage/microglia toward the M2 phenotype and accordingly stimulate them to become anti-inflammatory, while other glioma-derived factors, including MCP-1 and VEGF, can recruit myeloid cells into the tumor site. The latter further promote tumor expansion by secretion of molecules that favour angiogenesis, tumor growth and invasion. Reproduced with authorization of Oxford University Press, obtained form [11].

2.4.5.5. Glioma-associated T cells

In the 60's Bertrand et al, were the first to characterize glioma infiltrated lymphocytes [183]. The infiltration was heterogeneous from patient to patient and the lymphocytes were mainly localized in perivascular areas [184, 185]. The study of glioma infiltrating T cells gained prominence during the last three decades. This research revealed that the majority of tumor

infiltrated lymphocytes are T cells [186-188]. Furthermore, this infiltration increased with tumor grades, and the degree of infiltration was associated with increased permeability of BBB [189]. The majority of T cells were Th-2 polarized, exhibiting high expression of IL-4 and GM-CSF mRNA [188]. The isolated tumor infiltrated T cells presented low proliferation, cytokine secretion and cytotoxic capacity [187, 188, 190-192]. Furthermore, the expression of CD4 and CD8 was variable depending on the tissue specimen inspected [191, 193-195]. Overall, this variability between studies could be attributed to differing methodologies. As a result, the correlation between the degree of glioma lymphocyte infiltration and survival is often controversial between studies [196]. The analysis of glioblastoma biopsies revealed also the presence of regulatory T cells (Treg), $CD4^+CD25^+Foxp3^+$ [164, 197-199]. While the presence of these Treg populations correlated with tumor grades of malignancy [200], the cells were not abundant and not associated with GBM patient outcome [189]. As observed for infiltrated myeloid cells, glioma secreted factors play a central role in T cell recruitment and polarization as recently proposed for TGF-β [189].

2.4.5.6. Glioma-associated Natural Killer (NK) cells

Studies specifically investigating NK cells associated with glioblastoma are really scarce. In PAPER I, we compiled the existing articles about different brain tumors, including GBM.

2.4.5.7. Glioma-associated impaired systemic immunity

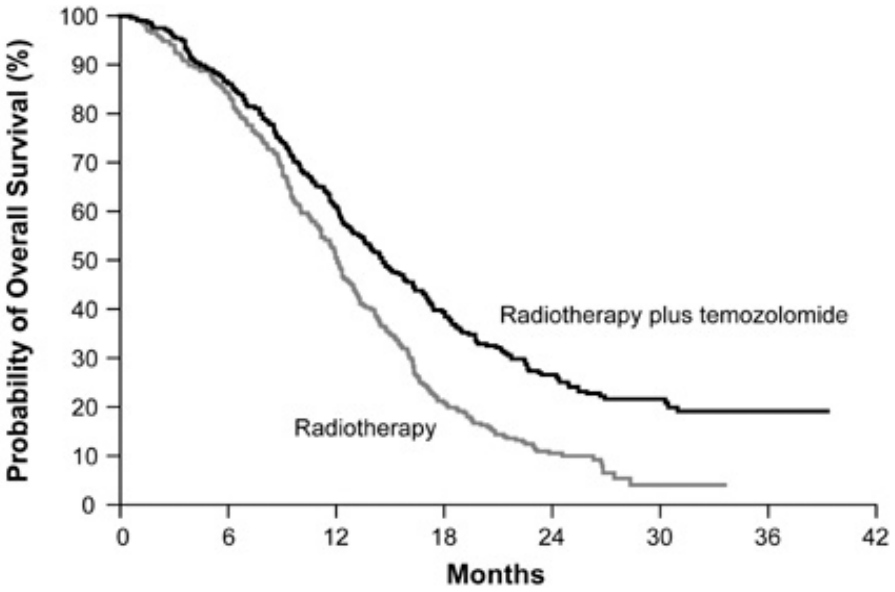
GBM patients commonly present with systemic immunosuppression associated with a reduction of peripheral PBMC count, associated with GBM mediated immunosuppression [201] but also the anti-cancer therapy received [202, 203]. Authors reported reduced T cell numbers and modification of their functions [204-206] associated with their phenotypic changes [197, 203, 207]. In contrast, the number of Treg cells was increased in patients compared to normal donors [208]. Increased Treg numbers are correlated with diminished peripheral monocytes and their altered phenotype, including decreased MHC class I and class II molecules expression and reduced antigen presenting cell properties in GBM patients [209, 210]. Furthermore, the glioma-associated monocytes secreted higher levels of EGF [211] and IL1-β [212] compared to normal donors, both factors that favor tumor promotion [213]. NK cells were also shown to be altered in their phenotype and function in patients with glioma [214]. These modifications of the systemic immunity were associated with glioma derived factors that modulate the host cells to drive tumor progression.

2.5. Current treatment of GBM patients

The current treatment of GBM patients is considered more palliative than curative, as the disease is lethal with virtually no long term survivors. Current standard care for GBM was set after randomized double-blinded multicenter phase III study from the EORTC_NCIC [3] and consists of maximal surgical resection, followed by radiotherapy and concomitant and adjuvant chemotherapy with temozolomide (TMZ). Furthermore, patients received additional treatment for symptoms related to glioma, such as steroids to relieve edema, anticonvulsants in patients with history of seizures and anti-depressants.

Without treatment the GBM patients die within 3 months, while the patients' median survival is improved by 3 months following maximal surgical resection. The major improvement in patient survival was the introduction of the TMZ chemo-radiation regimen that improved the median survival of patients from 12.1 months with radiotherapy alone to 14.6 months with TMZ (Figure 10)[3].

Figure 10: Kaplan-Meier estimates of overall survival of GBM patients according to treatment.



At a median follow-up of 28 months, the median survival was 14.6 months with radiotherapy plus temozolomide and 12.1 months with radiotherapy alone. Reproduced with permission of Massachusetts Medical Society, published in [3].

2.5.1. Neurosurgery

Patients with suspected GBM following MRI initially undergo surgical resection. However due to the infiltrative nature of GBM, the surgical resection is rarely complete and the GBMs recur within three months. Despite this recurrence, several randomized phase III clinical studies reveled a significant survival benefit following tumor resection in patients with GBM [3, 215, 216]. Furthermore, extensive resection also increased the efficiency of temozolomide chemoradiation [3, 216]. In parallel, surgery is essential to decrease neurological focal symptoms caused by the tumor and its surrounding edema. The extent of surgical resection will depend on the age of the patients, the size of the tumor, especially its localization within the brain and the patients' performance status, measured by the Karnofsky Score. The risk for surgical complications will govern the possibility of doing a near-total resection [217].

2.5.2. Radiotherapy

External-beam ionizing radiotherapy is administered concomitantly with Temozolomide chemotherapy 5 days a week given as 2 Gy daily doses, amounting to a 60 Gy full dose over 6 weeks [3]. While, the radiotherapy following surgery was demonstrated to prolong overall

survival of GBM patients, the GBM responsiveness is variable [218]. Following tumor recurrence, selected patients could undergo stereotactic re-irradiation by focused gamma radiosurgery (gamma knife) [219]. In addition, some clinical centers also use brachytherapy for recurrent GBM patients. This technique involves insertion of radioactive iodine 125 (^{125}I) seeds in the recurrent GBM bulk to deliver additional dose while sparing healthy tissue. This method provides encouraging survival rates with relatively low complication rates and a good quality of life [220].

2.5.3. Chemotherapy

Temozolomide is now the major chemotherapeutic agent used to treat GBM patients. This drug is an alkylating agent that has the advantage of crossing the blood brain barrier following oral delivery. The drug is given orally during radiotherapy (75 mg/m^2 per day) and after radiotherapy for 6 cycles of administration for 5 days every 28 days (150-200mg/m^2 per day) [3]. While this concomitant administration of TMZ to radiotherapy was described as the cause of major improvement of GBM patients' survival, this drug is effective mainly in the group of patients presenting with methylated promoter for the gene O6-methylguanine-*DNA methyltransferase* (*MGMT*). Thus, methylation of this promoter permits an epigenetic silencing of this gene coding for the DNA repair enzyme, which thereafter can limit the resistance to alkylating chemotherapy. Approximately 50 % of all GBM patients harbor a methylated MGMT promoter and thus respond poorly to TMZ chemotherapy. Nevertheless, patients treated with TMZ chemotherapy relapse and there is no effective alternative therapy after tumor progression/recurrence [221]. That is why there is still an urgent need for the development of new therapeutic strategies and molecular targets for GBM patients.

2.6. Immunotherapeutic strategies for glioma

A major effort has been dedicated to the identification and characterization of novel and reliable glioma associated antigens (GAA) that can then serve as powerful tools for tumor subtyping, diagnosis and immunotherapeutics. Despite this, relatively low amounts of GAA have been characterized so far, especially in comparison to other solid cancers like melanoma [196]. The important role played by cytotoxic T lymphocytes (CTL) in tumor rejection has focused the attention on the discovery of GAA that are targets for CTL. On the other hand, several studies reported specific humoral immune responses directed to astrocytoma specific antigen [222, 223]. Several antigens were characterized using serological analysis of recombinant cDNA expression libraries (SEREX), where diluted serums from cancer patients were used to detect tumor antigen [224]. Finally, several studies demonstrated the existence of glioma molecules that could be immunogenic for innate immune cells, such as NK or NKT cells, that could distinguish between neoplastic and normal cells and that could be useful for the development of immunotherapy [225, 226]. In this section we will review the different immunotherapeutic strategies in development or in clinical trial for the care of GBM patients.

2.6.1. Active immunotherapy or tumor vaccines for glioma

Cancer active immunotherapy attempts to activate the intrinsic immune system against the tumor cells. This activation of immunity could be non-specific by modulation of the general immune response using cytokines, bacterial proteins or other cell signaling components. On the

other hand, specific active immunotherapies focus on a specific tumor associated antigen in order to generate humoral or cellular immune responses. Multiple sources of antigens could be used for active immunotherapy such as tumor purified antigenic peptide, tumor-derived mRNA, peptides eluted from tumor MHC class I molecules, and synthetic peptides. Furthermore, these antigens can be injected alone or in association with adjuvants or through the intermediate of DCs that are considered to be essential for the development of a potent immune response [227].

2.6.1.1. Active immunotherapy using whole glioma cell vaccines

Initially, vaccine strategy to treat glioma patients consisted of the inoculation of inactivated, autologous glioma cells. The advantage of this method is that it could immunize the patient for a panel of multiple GAA naturally expressed by glioma cells. However it may also increase the risk of a subsequent development of autoimmune encephalomyelitis [228] since some of the antigens may be shared by components of the normal brain. Up to now 9 patient trials focused on GBM treatment were reported [229-236]. These trials constitute 1 phase I clinical trial [236], 1 phase I/II clinical trial [237], 5 pilot studies [230, 231, 233-235] and 2 case reports [229, 232]. The inactivation of autologous tumor cells was mainly done by radiation, but formalin fixed cells were also used [235, 237]. Several teams employed genetically modified autologous tumor cells before inoculation for secretion of IL-2, IL-4 or GM-CSF [229, 232, 234], while others used virus infected tumor cells [230, 233] in the goal to enhance their immunogenicity. Subcutaneous or intradermal inoculation of these tumor cells did not cause severe adverse effects, and clinical responses were associated with modestly increased survival in 6 studies [231-233, 235-237].

2.6.1.2. Active immunotherapy using DC

The DCs are considered as professional antigen presenting cells (APC) with a fundamental role in eliciting, maintaining and regulating adaptive T cells response [227]. In the particular case of glioma, increasing numbers of clinical studies demonstrate the safety and preliminary efficiency of cellular vaccine using antigen pulsed DCs [238]. Actually, there are 16 clinical trials reported so far, with 9 phase I clinical trials [239-247], 6 phase I/II trials [248-253], and 1 phase II trial [254]. There are several variations between the studies, as each team developed its own method for the preparation and maturation of DCs. Furthermore, the route of DC administration is typically intradermal, but could also be subcutaneous or intramuscular and more rarely intratumoral. Moreover, the sources of GAA also differed between the studies from autologous tumor lysate to the specific GAA. All these particularities are summarized in the review of Xu et al. [238].

2.6.1.3. Active immunization using bacterial or viral products

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are a potent inducer of both innate and adaptive immunity that drive the immune response towards the Th-1 phenotype [255]. These molecules represent one of the most studied bacterial products tested so far for treating glioma and so far, promising data have been obtained in all syngeneic murine glioma models tested [256]. Indeed in a syngeneic rat model of GBM the intracranial inoculation of CpG-ODNs increased survival of 85 % of animals. Interestingly,

several animals presented long term immunity against a new tumor challenge [257]. This beneficial outcome following intracranial inoculation of CpG motifs was also observed in mouse models and seems to be mediated by TLR9 [258] and induction of NK cell response [259]. A clinical trial evaluating the efficiency and the safety of intratumoral injection of CpG-ODNs to treat GBM was also performed. It includes 1 phase I clinical trial conducted in 24 patients with recurrent GBM. The injection of CpG motifs was done in non-resected tumors by convection—enhanced delivery (CED). It was well tolerated and was associated with 2 minor radiological responses. Moreover the median survival appeared slightly greater than would have been expected in this population of patients [260]. Then, in a subsequent phase II trial, a population of patients presented a beneficial outcome following the treatment with increased overall survival [261].

Alternatively, viral like products such as polyinosinic-polycytidylic acid (poly I:C) are also under evaluation as candidate for active immunotherapy for glioma. Poly I:C is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. It is a TLR3 agonist known for its ability to induce inflammatory immune responses via NFκB pathway [262]. One pilot study demonstrated the safety and the prolonged survival of GBM patients following several intramuscular injections of poly I:C [263]. The beneficial outcome of patients receiving poly I:C associated with radiation was also observed in 2 independent phase II clinical trials without [264] or with adjuvant temozolomide treatment [265].

2.6.1.4. Major human glioma antigen candidates for active immunotherapy

2.6.1.4.1. IL-13Rα2

IL-13Rα2 is a membrane glycoprotein shown to be expressed by almost 80 % of GBM samples tested by immunohistochemistry and immune fluorescence [266], but not by cells from normal tissues except in the testes [267] and only weakly expressed by normal astrocytes [268]. Its function was recently characterized in an animal model, where it was reported that the presence of a unique IL-13Rα2 decoy receptor prevents GBM apoptosis following IL-13 ligation [269]. This finding increased the appeal for this molecule for targeted therapy [270]. Furthermore, epitopes derived from IL-13Rα2 could serve as attractive components for peptide-based vaccines for GBM patients [247, 271].

2.6.1.5. The erythropoietin-producing hepatocellular carcinoma A2 (EphA2)

EphA2 is a tyrosine kinase receptor shown to be elevated in approximately 90% of GBM specimens but virtually absent from normal brain [272, 273]. During embryonic development EphA2 is present in the nervous system, but there is no expression in the normal adult brain [274, 275]. It is observed at the point of cell to cell contacts linked by its ligand ephrinA1 [276]. Interestingly, EphA2 overexpression was correlated with poor patients' outcome [275, 277]. Indeed this molecule was implicated in carcinogenesis [278], tumor cell migration, invasion [279] and angiogenesis [280, 281]. The immunization of HLA-A2 transgenic mice with EphA2 peptide resulted in the development of an epitope-specific CTL response in splenocytes [273, 282].

2.6.1.6. Wilm's Tumor (WT) 1

WT1 is the protein product of the Wilm's tumor gene and is involved in the development of gonads and kidney before birth. Moreover, WT1 is a transcriptional factor for genes involved in cellular growth, differentiation and apoptosis [283]. Furthermore, the overexpression of this molecule was implicated in the malignancy of several types of cancer, such as acute leukemia, breast and others [284, 285]. This protein was shown to be expressed by 94 % of patient GBM samples [286] and was predominantly associated with high glioma grades (grades III-IV) [287]**.** It is a GBM survival factor promoting tumorigenicity [288], chemoresistance [289] and invasiveness [290]. Peptides generated from WT1 proteins were previously shown to induce potent CTL responses [291]. Furthermore, a phase II clinical trial of vaccination using HLA-A24-restricted WT1 peptide demonstrated complete and partial responses in 9.28 % of patients with recurrent GBM and median progression-free survival of 20 weeks [292].

2.6.1.7. Sry-Related High-Mobility Group Box (SOX)

SOX genes encode a family of transcriptional factors that play an important role during embryogenesis and the development of several tissues [293]. The expression of SOX family members was frequently observed in tumor samples and cell lines [294]. Glioma biopsies exhibit increased expression of SOX2 [295], SOX5 [296], SOX6 [297] and SOX11 [298]. Experimental evidence indicated that SOX2 gene is a regulator of glioma cell proliferation and tumorigenicity [299, 300]. SOX5 and SOX 6 are expressed in GBM samples but only faintly in the normal adult brain [296, 301]. Specific IgG against SOX5 and SOX6 were found in 30 % of patients' sera [296, 297]. Furthermore, the presence of IgG directed against SOX5 was shown to be correlated with better patient survival [296]. On the other hand, vaccination with DNA or peptides derived from SOX6 was shown to be a potent inducer for CTL responses *in vivo* [302, 303]. SOX2 and SOX11 derived peptides could also be potential candidates for peptide vaccination as they were able to lead to stimulation of HLA-A2 restricted CTL against GBM cell lines [295, 304].

2.6.1.8. Human epidermal growth factor receptor 2 (HER-2/neu)

HER-2/neu is a member of the EGFR family encoding a transmembrane glycoprotein with intracellular tyrosine kinase activity. This receptor is frequently overexpressed in various tumors, including GBM, and was shown to regulate tumor proliferation, survival, migration, adhesion and angiogenesis [305]. Its expression correlated with the degree of astrocytoma anaplasia [306] and was expressed by 20 to 90 % of GBM biopsy tissues and cell lines [307-310], but not in adult CNS [311]. Overexpression of HER-2/neu is a marker for poor prognosis in GBM patients [309]. However, in 2007 a study on 44 GBM patients did not observe HER-2/neu expression by gene amplification by fluorescence in situ hybridization (FISH) [312]. Nevertheless, despite these conflicting results, the potential overexpression of HER-2/neu in GBM and its recognized immunogenicity for T cells, make it a potential target for T cell mediated immunotherapy [313]. Moreover a peptide derived from HER-2/neu was shown to be a potent inducer of HLA-A2 restricted CTLs against GBM cell lines [310]. Furthermore, HER-2/neu is one of selected peptides that constituted a new DC-pulsed vaccine showing promising therapeutic potential for GBM patients [247].

2.6.1.9. Epidermal growth factor receptor (EGFR) and its type III variant (EGFRvIII)

EGFR is a transmembrane tyrosine kinase receptor that plays an important role in cellular growth and differentiation in healthy tissues, but also promotes tumorigenesis and malignancy [314]. EGFR gene amplification, rearrangement, and overexpression are some of the most frequent genetic alterations associated with GBM patients [315]. Moreover, EGFR amplification is often correlated with the expression of the mutant EGFR gene, EGFRvIII [316, 317]. The latter, is characterized by the gene deletion of the exons 2 to 7, resulting in a truncated extracellular domain with ligand independent constitutive activity [318]. However this mutated form of EGFR is not expressed by normal cells and may be a preferable target for immunotherapeutic strategies [319]. EGFR is overexpressed in approximately 40 % of GBM biopsy tissues [315, 320, 321] and in this EGFR positive GBM almost 70 % were shown to express EGFRvIII [317]. Several studies demonstrated that EGFRvIII overexpression correlated with increased proliferation and radioresistance of neoplastic cells [322, 323]. The presence of these receptors in GBM biopsy tissue was a prognosis of poor outcome for the patients [324, 325].

EGFRvIII peptide-pulsed DCs were able to stimulate CTLs *in vitro* against HLA-A0201+ EGFRvIII transfected U87 glioma cells [326]. In 2008, a phase II clinical trial demonstrated that the vaccination with peptides derived from EGFRvIII was able to induce potent T- and B cell immunity in treated GBM patients and was associated with prolonged survival. However, all recurrent tumors had lost the EGFRvIII expression suggesting the development of an antigen-loss variant [327].

2.6.1.10. Squamous Cell Carcinoma Antigen Recognized by T Cells (SART)

Schichijo et al. were the first to identify SART-1 by screening cDNA of carcinoma on HLA-A2 restricted CTLs. They characterized its immunogenicity for activation of CTLs against carcinoma cell lines [328]. Then SART-1 was identified in various tumors, including glioma where it is expressed by 72 % of glioma cell lines and 50 % malignant glioma [329]. This research team demonstrated that this antigen could be a target molecule for specific immunotherapy of patients with brain tumors expressing HLA class I antigens [329]. Recently the breast cancer associated allele of rs660118 SNP in the gene SART1 was shown to be of near doubled frequency in glioblastoma patients, indicating that this SNP plays a role in the immunogenicity of this gene [330]. SART-3 expression was also identified in 91 % of glioma biopsy tissues and in 100 % of glioma cell lines analysed, but absent from normal brain tissues tested [331]. In addition it was also a potent source of peptides with potential for the induction of CTL responses against STAT3-expressing glioblastoma cell lines [331].

2.6.1.11. Tyrosinase-related protein (TRP-2)

TRP-2 is a glycoprotein normally expressed in human pigmented melanocytic cells and melanoma, implicated in melanin production [332]. It was reported that TRP-2 overexpression was associated specifically with resistance to DNA damaging drugs and radiation treatment [333, 334]. However, RT-PCR analysis demonstrated that TRP-2 was present in 51.2 % of primary tumor cell lines derived from GBM patients and that it was a potent source of immunogenic

peptides for pulsed DCs or peptide-based vaccination [335]. Then the same team demonstrated that patients vaccinated with DC-pulsed with TRP-2 peptides responded better to subsequent chemotherapy, leading to improved outcomes [336]. Furthermore, this antigen was included in the multi-epitope-pulsed DC vaccine for patients with newly diagnosed glioblastoma in phase I trial that recently demonstrated beneficial outcomes in GBM patients [247].

2.6.1.12. Absent in melanoma (AIM-2)

AIM-2 antigen is expressed in a wide variety of tumor types but also in normal tissue and 90 % of GBM cell lines were positive by RT-PCR [337]. This molecule is implicated in tumorigenicity and control of cellular proliferation [338]. It was also recently identified as a major regulator of DNA-mediated inflammatory responses [339]. Peptides derived from AIM-2 have been tested for their capacity to induce CTL activation against GBM cell lines expressing it under the restriction of HLA [337]. Furthermore, AIM-2 is also a constituent of the peptides mixture in the successful clinical trial phase I in DC-pulsed vaccine for GBM [247]. However, as this molecule could be expressed by normal cells it could lead to toxic effects when used as a target for immunotherapy.

2.6.1.13. Melanoma-associated antigen (MAGE)

MAGE, initially identified from melanomas, was the first identified tumor antigen recognized by T cells [340]. This molecule is expressed by a variety of malignant cells, but its expression is limited to testes, placenta and brain in normal tissues [340, 341]. Its expression in malignancies was associated with aggressive clinical course, poor clinical outcome and the resistance to chemotherapy [342, 343]. mRNA expression was characterized in GBM cell lines and tissues [344-347]. The high expression of MAGE-1 by GBM cell lines was shown to be a potent inducer of IFN-γ secretion by antigen specific CTLs [310]. Furthermore, it is also a constituent of the peptides mixture used in the successful phase I clinical trial of DC-pulsed vaccine for GBM [247]. However as this molecule could be expressed by normal cells it could lead to toxic effects when targeted for immunotherapy.

2.6.1.14. Human melanoma-associated antigen (Gp100)

Gp100 is a melanocyte differentiation antigen recognized in patients with melanoma by HLA-restricted CTLs and antibody [348, 349]. It was characterized in some GBM tissue samples and cell lines [266]. This molecule is detectable by RT-PCR in normal brain tissue [346]. It was shown that gp100 specific CTLs were able to produce IFN-γ and to be cytotoxic in contact with gp100 positive glioma cell lines[266, 282, 310]. Furthermore, it is also a constituent of the peptides mixture with successful phase I clinical trial of DC-pulsed vaccine for GBM [247]. However as this molecule could be expressed by normal cells it could lead to toxic effects when targeted for immunotherapy.

2.6.1.15. Cytomegalovirus (CMV) associated antigens

In 2002, Cobbs et al. were the first to characterize the high incidence of patients with malignant gliomas infected by CMV (more than 90%), as well as the multitude of CMV gene

products expressed by these tumors (IE1, pp65, and late antigens). This expression of CMV products was shown to be restricted to GBM tissues as normal brain and patients with non-malignant tumor were negative for CMV proteins [350]. These observations were further confirmed by several research teams [351-353]. The implication of the CMV infection in modulation of oncogenic phenotype of GBM started to be explored in detail and it was reported that IE1 mediated mitotic potential [354]. Later, the expression of US28 CMV protein in glioblastoma was characterized and its possible implication in the promotion of an invasive and angiogenic phenotype of GBM was proposed [355]. While the specific role for CMV in development of GBM needs further analysis, many research teams agree on its potential as a novel target for a variety of therapeutic strategies. Prins et al., reported a case report where the vaccination of DC-pulsed with autologous tumor lysate of a patient resulted in a rapid mobilization of CMV-specific CTL responses to the pp65 CMV immunodominant epitope [352]. This highlights the potential for targeting CMV by DC-based vaccination strategies as an adjunct to standard treatments for glioblastoma. So far, several clinical trials are under investigation, see www.clinicaltrials.gov. Phase I/II immunotherapy clinical trial of autologous CMV pp65 RNA loaded DCs was initiated (ATTAC Protocol- FDA-IND-BB-12839; Duke IRB Protocol 8108; PI: Duane A. Mitchell). Initial results are highly encouraging. Patients exhibited a median progression-free survival (PFS) of 15.4 months and an overall survival (OS) of 20.6 months. Both outcomes are highly significant compared to matched historical controls ($p = 0.004$) [356].

2.6.2. Passive immunotherapy

2.6.2.1. Antibody based therapy

There is a multitude of antibody-based therapies in development, as antibody-based inhibition strategies or radioimmunotherapy.

2.6.2.1.1. Antibody-based inhibition strategies

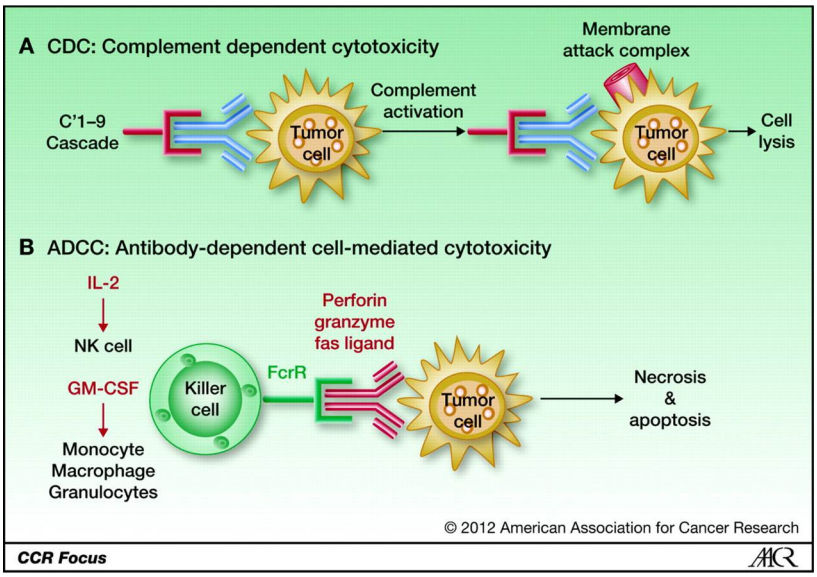
This section will focus on the monoclonal antibody raised against extracellular GBM molecules known to be implicated in tumor physiology. The antibody can be employed to manipulate the host immune system to recognize and kill labeled cells by Fc receptor positive cells by processes called complement derived cytotoxicity (CDC) and/or antibody dependent cellular cytotoxicity (ADCC) (Figure 10).

The most studied targets for antibody based therapy are undeniably the EGFR and its mutant EGFRvIII [357]. The treatment with mAb against EGFRvIII of mice bearing syngeneic tumors transfected with a murine homolog of the variant receptor increased survival [358]. However, clinical trials using cetuximab to treat GBM patients gave overall disappointing results despite the fact that the mAb was well tolerated [359]. A phase I/II clinical trial study of the efficacy of combining radiotherapy, TMZ and cetuximab, to treat patients with primary GBM was conducted in 46 patients [360]. However, the results were not yet published. Other anti-EGFR monoclonal antibodies are in initial stages of clinical development for GBM, and include panitumumab and nimotuzumab [357, 361]. Furthermore, passive immunotherapy using monoclonal humanized antibody against HER2/neu could be conceivable as Herceptin® was able to induce apoptosis and cellular-dependent cytotoxicity of HER2/neu-expressing GBM cell lines [362]. Several other antibodies have shown promising results following clinical

investigation for antibody based therapy for GBM patients such as Bevacizumab, directed against VEGF as monotherapy[363-366] or in combination with radio- and chemotherapy [367-369]. Pritumumab, a mAb directed at a tumor-specific variant of vimentin and anti-EGF also has shown promising clinical results in patients [370, 371].

Alternatively, the monoclonal antibody may also serve to disrupt receptor-ligand binding [372]. The application of this approach to glioma presents several limitations, such as the ability of the antibody to breach the BBB and then to reach the tumor core in the context of elevated interstitial pressure [373]. Moreover, there is the risk of development of cerebral autoimmune disease, leading to severe neurotoxicity [374].

Figure 10: Schematic representation of mechanisms of antibody-mediated tumor destruction.



Tumor cell destruction by (**A**) CDC and (**B**) ADCC. **(A)** CDC: antibody attaches to its target and initiates the complement cascade, resulting in the development of a membrane attack complex that makes the cell membrane permeable, causing cell lysis and death. **(B)** ADCC: following the recognition of its antigen by the paratop of the antibody, the Fc fragment of the monoclonal antibody is then recognized by the Fc receptors on monocytes, macrophages, granulocytes, and NK cells. These cells in turn may destroy neoplastic cells by antibody mediated phagocytosis or release of cytotoxic granules. Reproduced with authorization of American Association for Cancer Research, illustration from [6].

2.6.2.1.2. Radioimmunotherapy (RIT)

As an alternative strategy, monoclonal antibody delivery may refine targeted delivery of cytotoxic molecules such as radionucleotides in GBM therapy. The advantage of using antibody conjugated to radionucleotide is double, as it could serve for both imaging and for targeted radiotherapy. The advantage of this technique compared to classical radiotherapy is that RIT concentrates radioactivity at the tumor site protecting normal brain tissue from adverse effects. Two types of radionucleotides are used, α particles, such as ^{211} Astatine (At) and β particles, such as ^{131} Iodine (I) and ^{90} Yttrium (Y) [375]. The most studied target for RIT is tenascin-C, which is a glycoprotein expressed on several tumor types including gliomas. Its expression was correlated with advancing tumor grade and almost 90% of GBM express it at high levels [376].

Several clinical trials were performed using anti tenascin-C antibody clone 81C6 coupled to ^{131}I. These studies demonstrated that inoculation of this antibody in the tumor resection cavity following surgery increased median survival time of patients with recurrent and newly diagnosed GBM [377-380]. The anti-tenascin mAb coupled with ^{211}At also improved survival times, where 2 patients out of 14 presented survival of almost 3 years [381].

2.6.2.2. Coupled Target Toxins

The specific cytotoxicity of neoplastic cells could also be achieved using cytokines coupled to a toxin, also called immunotoxins [382]. One of these cytokines is IL-4 coupled to *Pseudomonas exotoxin* (PE). The IL-4 receptor was shown to be up regulated in glioma cells in comparison to normal tissues [383]. Puri et al. developed the chimeric fusion protein [384], which was shown to be well tolerated by patients in phase I studies [385]. As already mentioned IL-13 receptor is also a good target for immunotherapy. A recombinant protein consisting of IL-13 and a truncated form of PE was tested in Phase I-III clinical trials. Despite the high expectation for this approach, the Phase III trial failed to achieve clinical endpoints for toxicity, due to normal cells bearing the physiologic receptor for IL-13 [386].

2.6.2.3. Cellular based therapy

2.6.2.3.1. LAK cells based therapy

The research on adoptive transfer of immune effectors to treat glioma patients is slowly progressing. Historically, lymphokine-activated killer (LAK) cells were the first developed cellular therapy to treat GBM patients. The efficiency of LAK cells was investigated by 12 clinical trials in the literature , 1 pilot study [387], 6 phase I clinical trials [388-393], 5 phase I/II trials [394-398]. LAK cells were generated *ex vivo* from culture of patients' blood lymphocytes in presence of IL-2 cytokine for several days. However, LAK cells are a mixture of cells with tumor killing properties, constituted mainly by CTL and NK cells. These cells were often used as an adjunct to surgery and mainly in association with low dose of IL-2. From 1 to 15 injections were performed into the brain of patients with recurrent GBM. In 4 clinical trials, treatment was well tolerated and overall survival was increased [395-398]. Taken together, these trials presented 5 complete responses, 13 partial responses and 6 stable diseases in a total of 118 patients, but they did not show a significant correlation between the clinical results and radiological response [394]. Nonetheless due to the difference in clinical designs it is difficult to accurately compare these trials. Especially because some studies included patients totally devoid of chemotherapy or corticosteroid treatment to avoid immunosuppression following immunotherapy [388, 391, 397, 398], while other studies demonstrated no influence between drug therapy and the generation and the lytic activity of the effector cells [389, 392, 394]. Nonetheless, in a case report, a young adult with recurrent malignant glioma achieved a complete remission following LAK treatment with low dose of IL-2 [399].

2.6.2.3.2. NK cells based therapy

Despite the promising results in modulating NK cell response against brain tumor in animal models [400, 401] and the fact that these cells are considered suitable candidates for adoptive immunotherapy of both hematological and non-hematological malignancies [402, 403],

only one clinical study, to our knowledge, examined their therapeutic potential for GBM patients [404]. The authors demonstrated feasibility of NK cell based immunotherapy in glioma patients. The NK cells were obtained after culture of PBMC with irradiated human feeder cells and IL-2. Following several runs of intracranial plus intravenous NK cell inoculation this protocol presented no severe toxicity and in a total of 9 patients, 3 presented partial responses and 2 minor responses; with respectively 50 % and 25 % decrease in the total tumor volume that persisted for four weeks [404].

2.6.2.3.3. T cells based therapy

In contrast, several phase I clinical trials to treat gliomas using intracerebral injection of CTL purified from patient's PBMC have been performed [405-408], and 1 pilot study using tumor infiltrated lymphocytes (TIL) [409]. Other phase 1 and pilot studies tested the intravenous adoptive transfer of the autologous CTL [410-414]. These studies used from 1 to 13 injections of cells per patient. Again, these T cell based immunotherapies were well tolerated by patients. 10 trials have reported 18 cases of stable disease, 28 partial responses and 3 complete remissions among 95 patients treated. Four studies have even been able to demonstrate a benefit in survival. Thus, on face value, T cell therapies achieved higher rate of positive responses compared to LAK therapies in patients.

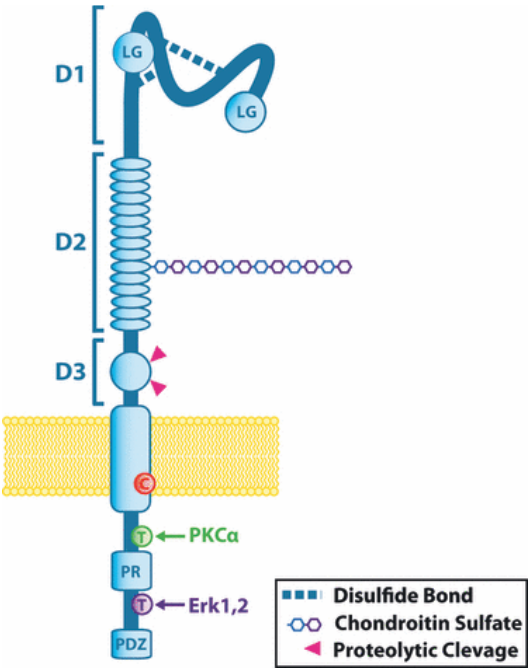
2.6.2.3.4. Chimeric antigen receptors (CARs) engineered immune cells

An interesting, recent innovative technology consists of engineering a chimeric molecule that has the antigen binding domains of a monoclonal antibody fused with a signal transduction domain of CD3. These constructs are called chimeric antigen receptors (CARs) [415]. The main advantage of cytotoxic cells (CTL or NK cells) genetically modified to express CARs is that the antigen recognition is not restricted to MHC class I molecule expression by target cells. CARs have been used for targeting several malignancies, including gliomas [416, 417] [418]. Indeed, intratumoral injection of IL-13Rα2-specific CAR T cells in 3 patients with GBM, concluded that while the administration was safe and tumor responses were observed, the tumors recurred and these neoplastic cells were negative for IL-13Rα2 [417].There are promising *in vivo* studies investigating EphA2-specific CAR T cells for targeting and killing of EphA2-positive GBM [418], and EGFRvIII [419].

2.7. NG2/*CSPG4* as a new target for immunotherapy

The neural progenitor Neuron Glia-2 (NG2) is a chondroitin sulphate proteoglycan (CSPG) encoded by the *cspg4* gene on chromosome 15, henceforth denoted NG2/*CSPG4*. This is a single 330 kDa transmembrane glycoprotein presenting a large extracellular domain and a short cytoplasmic tail (Figure 11). This glycoprotein was first characterized in rat by Stallcup et al. [420] and then its primary structure was elucidated for rats [421], mice [422] and humans [423] indicating that it is evolutionarily conserved. The human NG2/CSPG4 was first characterized as melanoma cell surface proteoglycan (MCSPG) [424, 425]. This proteoglycan represents the therapeutic target in this thesis.

Figure 11: Structure of the NG2/*CSPG4* proteoglycan.



The extracellular domain of this proteoglycan consists of 3 subdomains. D1 is a N-terminal globular domain consisting of laminin G-type regions (LG) and disulfide bonds. D2 subdomain is formed by 15 repeated CSPG. The D3 globular subdomain contains sites for N-linked carbohydrate modification, binding sites for lectins and proteolytic cleavage by MMPs or other proteases. The transmembrane region of CSPG4 contains a cysteine residue (C) at position 2230 that may that may play a role in membrane localization. The intracellular domain contains tyrosine residues (T). The proline-rich region (PR) may comprise a non-canonical SH3 protein interaction domain, and the C-terminus contains a 4 residue PDZ domain-binding motif (PDZ). Reproduced with authorization of John Wiley and Sons, published in [8].

2.7.1. Structure and partner molecules of NG2/*CSPG4*

The first extracellular domain (D1 illustrated in Figure 11) is characterized by the presence of 2 Laminin G (LG)/ Neurexin/ Sex Hormone Binding Globulin (LNS) domains in N-terminal that place NG2/*CSPG4* in the family of neurexins. The latter are cell adhesion molecules implicated in synaptic functions [426] and angiogenesis [427]. However, to date there is no cell adhesion ligand recognized to bind the LNS domain of NG2/*CSPG4* [428]. The D2 subdomain consists of 15 repeated CSPG in human and in rodent that is a single chondroitin sulfate glycosaminoglycan (CS-GAG) chain, forming a binding site for soluble growth factor ligands or receptors (such as PDGFAA, FGF2 or PDGFα receptor [429]), β1-integrin, collagen IV and MMPs [430]. The number of CS-GAG chains is variable following the cell type and its maturation status [422]. The last extra cellular domain (D3) presents a binding site for protease cleavage near the transmembrane domain that could result in fragmentation and release of NG2/*CSPG4* that can be further detectable in the sera of both normal people and those with malignant disease [431]. The intracellular domain is short and presents a PDZ domain that can bind Multi-PDZ Domain protein 1 (MUPP1) [432], Glutamate Receptor Interaction Protein (GRIP) [433] and Syntenin-1 [434]. MUPP1 served as a linker for NG2/*CSPG4* with key structural and/or signalling components in the cytoplasm [432]. In the early stage of myelination, Syntenin-1 co-localized with NG2/*CSPG4* in oligodendrocytes at the synapses and in wrapping axons helping in their migratory function [434]. The interaction of NG2/*CSPG4* with GRIP has implications for glial-neuronal signalling [433].

2.7.2. Expression of NG2/*CSPG4* in normal tissue and malignancy

2.7.2.1. Expression of NG2/CSPG4 in normal tissue

There are a variety of cells in and outside the CNS expressing NG2/*CSPG4* in mammalians. The proteoglycan expression is more pronounced in non-differentiated stem cells and is reduced upon their maturation [431]. NG2/*CSPG4* is found in immature chondroblasts [435], bone marrow mesenchymal cells [436] and smooth muscle cells [437]. Moreover, epidermal and hair follicle progenitor cells were shown to express this molecule in adult and fetal human skin where it promotes stem cell clustering [438, 439]. Furthermore, NG2/*CSPG4* is implicated in the development of the vasculature [440] and was shown to be expressed by pericytes during neovascularization [441]. Thus, knock-out mice for NG2/*CSPG4* presented an abnormal vasculature [70]. In the nervous system, NG2/*CSPG4* is a marker for oligodendrocyte precursor cells (OPC) [430], characterized for their ability for self-renewal, proliferation and their capacity to further differentiate to oligodendrocytes, protoplasmic astrocytes and neurons *in vivo* [428, 430]. In adult rats CNS this proteoglycan could constitute up to 9 % of total cells [442] and during the differentiation of OPC the expression of the proteoglycan is lost [430]. Thus, NG2/*CSPG4* is not expressed by differentiated glial cells that are mature astrocytes and oligodendrocytes [430 803]. Indeed, the proliferative capacity of OPC could be reduced in adult NG2/*CSPG4* knockout mice [443].

2.7.2.2. Implication of NG2/GSPG4 in GBM malignant progression

NG2/*CSPG4* was characterized to be aberrantly over expressed in several cancers, such as melanoma [8], triple-negative breast carcinomas [444], malignant mesothelioma [445], soft tissue sarcoma [446], acute lymphoblastic leukemia [447], acute myeloid leukemia [448] and gliomas [69, 449, 450]. Several teams demonstrated that its expression correlates with poor clinical outcomes [446, 451-453] including in GBM patients [454]. NG2/*CSPG4* is more abundantly expressed by high grade glioma, such as GBM, compared to lower grade gliomas [69]. Indeed, this expression was often correlated with the level of glioma malignancy [69, 74, 455-457]. Moreover, animal models revealed that NG2/*CSPG4* positive OPC can sustain the tumor initiating mutation leading to gliomagenesis. These results implicated NG2/*CSPG4* positive OPC as cellular origin of glioma [458-461]. The proteoglycan is also expressed by tumor associated microvasculature [69, 462-464], and regulated glioma vascular morphology and function [449, 465]. Furthermore, *in vivo* implantation of NG2/*CSPG4* positive tumor cells in rats resulted in a higher microvascular density compared to their negative counterparts [465].

In addition to its role in angiogenesis, NG2/*CSPG4* is involved in the activation of several signalling pathways, including ERK and focal adhesion kinase (FAK), demonstrated to be critical for neoplastic cells motility, proliferation, and survival [466-468]. As already mentioned, the D2 domain of NG2/*CSPG4* (Figure 11) links the cells to the extracellular matrix, through interactions with β-integrin, permitting migration along blood vessels and nerves-fiber tracts [72, 469]. Furthermore, *in vitro* studies on melanoma revealed that specific antibodies directed against the proteoglycan could affect the attachment and spreading capacity of the cells [470, 471]. In addition, the proteoglycan is able to bind growth factors including PDGF-AA and FGF2 [429], that are critical for OPC proliferation [472-474]. We can suspect that GBMs that

highly express NG2/*CSPG4* can use these growth factors for their growth and angiogenic capacity. Furthermore, the implication of NG2/*CSPG4* in neoplastic growth is suspected as several teams reported proliferative advantage of glioma cells expressing this proteoglycan [450, 454]. Thus, the genetic signature of NG2/*CSPG4* positive GBM was highly associated with mitosis and cell cycling genes [475]. In addition, the proteoglycan is also critical for tumor survival following chemotherapy through activation of PI3K/Akt pathway in a β-integrin dependent manner [74]. Glioblastoma cells expressing NG2/*CSPG4* were also more resistant to ionizing radiation (IR) by mechanisms involving a rapid recognition of DNA damage following cycle checkpoint signaling [454]. This implication of NG2/*CSPG4* in the aggressiveness of GBM, such as the processes of tumor angiogenesis, cellular mobility, proliferation and survival make it an attractive target for directing therapeutic agents to treat GBM patients.

2.7.3. NG2/*CSPG4* as a therapeutic target

This subsection will summarize the experimental immunotherapy targeting NG2/*CSPG4* that has already been tested in several types of tumor models and patients. The majority of immunotherapies targeting the NG2/*CSPG4* were evaluated for treatment of melanoma. The first experimental trial targeting NG2/GSPG4 was done in the 80's using mAb9.2.27 against melanoma. This mAb was first tested alone or conjugated to diphtheria toxin in athymic nude mice bearing aggressive melanoma [476]. Surprisingly, both the native and diphtheria toxin conjugated mAb9.2.27 reduced tumor growth, indicating that host mechanisms could mediate mAb-dependent tumor suppression [476]. In the follow-up study, the same team demonstrated that the tumor suppressive property of mAb2.2.27 could be amplified by a co-administration of splenocytes pre-armed with the mAb9.2.27, through the mechanism of ADCC. Furthermore, they observed that while melanoma cells were resistant to the killing by unarmed effector cells, this resistance was abrogated following combination with mAb9.2.27 [477]. They further demonstrated that the ADCC was mostly performed by NK cells, as administration of splenocytes depleted of NK cells with anti-asialo GM1, failed to reduce tumor volumes *in vivo* [478]. The same mAb9.2.27 clone was administered to animals bearing human glioma for a targeted delivery of a vinblastine derivative to the tumor. This strategy delayed tumor growth compared to controls [479]. Furthermore, preclinical data using immunodeficient mice engrafted with NG2/*CSPG4* expressing human melanoma cell lines indicated that mAb directed against NG2/*CSPG4* can inhibit their growth, recurrence of disease, and/or metastasis [468, 480].

The potential of this proteoglycan as a target for immunotherapy gained clinical relevance when Mittelman et al. demonstrated, in a phase I/II clinical trial, that melanoma patients who developed antibodies against NG2/*CSPG4* following active specific immunotherapy presented increased survival outcomes [481, 482]. Additionally, regression of metastatic lesions in a few patients was evident [483]. Recently, humanized mAb against NG2/*CSPG4* was developed to overcome the potential side effects of using mouse mAb in patients [471, 484]. This humanized mAb diminished growth and migration of tumor cells *in vitro* and reduced growth of human tumor xenografts in immunodeficient SCID mice [471]. However, the immunological responses following active immunization using NG2/*CSPG4* derived peptide (HMW-MAA) indicate that this strategy requires further experimentation. Nevertheless, some results in animal models indicated that antigen specific CTLs raised against HMW-MAA are dependent on $CD4^+$ T cells expansion [485-487]. Moreover, the CTL response is not restricted to the neoplastic cells, as Maciag et al. demonstrated that this response could also

be directed against the tumor neovasculature by targeting pericytes expressing NG2/*CSPG4* [488].

The implication of NG2/*CSPG4* positive cells in the initiation, growth and infiltration of GBM make it a good target for immunotherapy. Nonetheless, the fine toxicological study may delineate the potential toxic effects of NG2/*CSPG4* targeting, as a minor population of normal brain cells express this molecule [442].

2.8. Natural killer cells

NK cells were first characterized as large granular lymphocytes that can kill virus transformed cells or tumor cells without restriction by the MHC molecules or prior activation. [489-491]. NK cells traffic through the body and were found in many organs, where they present a particular phenotype in relation to the organ microenvironment [492]. Furthermore, NK cell populations consist of several subtypes with particular functions. This section will go through human, mice and rat NK cells phenotypes and functions. Then, the biology of NK cells will be summarized. The particular feature of CNS associated NK cells will be exposed, as well as their implication in brain tumor. This section will end with the use of NK cells as a tool for immunotherapy.

2.8.1. NK cell phenotypes and functions

2.8.1.1. Human NK cells

In human, NK cells are characterized as $CD3^-CD14^-CD19^-$ lymphocytes, as non T cells, non monocytes and non B cells. Subpopulations of NK cells can then be separated following their level of expression of CD16 and CD56 (Figure 12A), with the two major populations $CD16^{neg}CD56^{bright}$ and $CD16^{bright}CD56^{dim}$ [493]. The $CD16^{neg}CD56^{bright}$ population is relatively low in peripheral blood where it represents up to 10 % of circulating NK cells, but is more frequent in lymph nodes, liver and CNS [493, 494]. These cells were recognized to have high secretory capacities of IFN-γ, TNF-α, GM-CSF, IL-10 and IL-13, but a relatively low cytotoxicity [493, 494]. $CD16^{bright}CD56^{dim}$ cells are found in majority in blood with 90 % of NK cells [493]. The latter population is mainly cytotoxic and presents marginal secretion events [493]. These functional specializations between subpopulations are reflected by their distinct phenotypes. Thus, $CD56^{bright}$ NK cells are devoid of the inhibitory receptors killer cell immunoglobulin-like receptors (KIR) as well as immunoglobulin-like transcript 2 (ILT2), but they express with high density the inhibitory receptor CD94/NKG2A compared to $CD56^{dim}$ NK cells [494, 495]. Moreover, both subpopulations present differential levels of chemokine receptors: while $CD56^{bright}$ NK cells highly express CCR_7 and $CXCR_3$, $CD56^{dim}$ NK cells arbor $CXCR_1$ and CX_3CR_1 [496, 497]. Furthermore, $CD56^{bright}$ NK cells express more adhesion molecules, such as CD62L, CD44 or CD49e than $CD56^{dim}$ NK cells which predispose them to home to secondary lymphoid organs [494]. The maturation processes from human NK cells are less documented than for mouse, and need further *in vivo* experimental evidence. Nonetheless, several data indicates that NK cells may mature from $CD56^{bright}$ to $CD56^{dim}$, in relation to their higher level of CD57 receptor expression on $CD56^{dim}$ cells that is a marker for terminal differentiation on human CTL and that is increased with age [498]. Furthermore, the existence of patients with accumulation of $CD56^{bright}$ in their peripheral blood, which may have NK cells

maturation defect [499]. In addition, as it is for mouse, human NK cells were shown to be distributed following the expression of CD11b and CD27, where $CD56^{bright}$ are mainly $CD27^{+}$, corresponding to the most immature subset of mouse NK cells [500].

2.8.1.2. Mouse NK cells

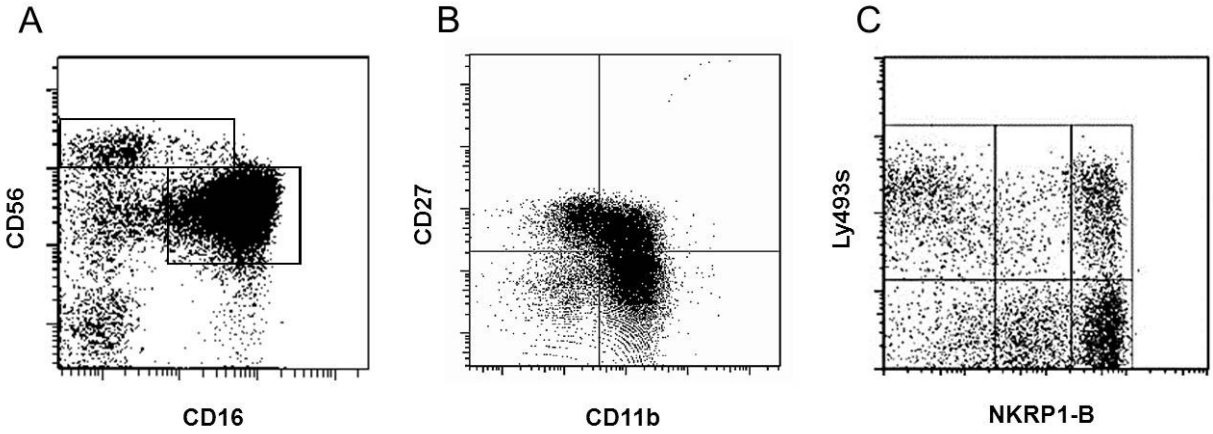
Mouse NK cells are characterized as non T cells expressing NK1.1 receptor, and phenotyped as $CD3^{-}NK1.1^{+}$ cells. They can be separated in 4 subpopulations following their expression of CD11b and CD27. It was described that the NK cell maturation proceeds from double negative $CD11b^{-}CD27^{-}$ cells to single positive $CD11b^{-}CD27^{+}$ cells, followed by double positive $CD11b^{+}CD27^{+}$ cells and finishing by a terminal differentiation status with single positive $CD11b^{+}CD27^{-}$ phenotypes [501, 502](figure 12B). Indeed, fetal and neonatal mice display more NK cells with $CD11b^{low}$ expression, while in adults these subpopulations are restricted to the bone marrow, lymph nodes as well as liver [503]. The more mature NK cell subtype with $CD11b^{high}$ phenotype is mainly found in spleen, peripheral blood and lung and its proliferative capacity is reduced [501, 504]. NK cells emerge from bone marrow and then undergo complex steps of maturation steps that further modulates their functions, [501] but also their dissemination through the body compartments [492, 505]. More mature NK cells with $CD11b^{high}$ phenotypes present higher expression of inhibitory receptors Ly-49C/I as well KLRG1, but a reduced expression of CD94/NKG2 receptors compared to $CD11b^{-}CD27^{+}$ population [502]. These differential phenotypes reflected also distinct cytotoxic and secretory capacity between the subgroups. The most immature form was more competent to secrete IFN-γ following IL-12/18 activation and to be cytotoxic against YAC-1 tumoral target, in comparison to the most mature NK cell subset [502].

2.8.1.3. Rat NK cells

The characterization of rat NK cells is mainly done as $CD3^{-}NKRP1A^{+}$ cells. Like NK1.1 in mouse, NKRP1-A is an activating receptor for rat NK cells [506]. The discrimination of rat NK cell subpopulations as shown for human and mouse remains elusive. The main improvement in the understanding of rat NK cell subsets came from the research of a Norwegian group that characterized subpopulations following the expression of Ly49s3 and NKRP1-B [5, 507, 508]. Ly49s3 is a stimulatory receptor and NKRP1-B is an inhibitory receptor that was firstly denoted as NKRP1-C [507, 509]. Inngjerdingen et al. characterized 4 subpopulations of rat NK cells: $NKRP1\text{-}B^{bright}Ly49s3^{-}$, $NKRP1\text{-}B^{bright}Ly49s3^{+}$, $NKRP1\text{-}B^{dim}$ and $Ly49s3^{+}$ (Figure 12C) [5]. These subpopulations presented a differential localization following the organs, where $NKRP1\text{-}B^{bright}Ly49s3^{-/+}$ subsets were predominant in Payer's patches, liver and peripheral blood but really low in bone marrow, spleen and inguinal lymph nodes. The phenotypic characterization of these cells by flow cytometry revealed that $NKRP1\text{-}B^{bright}Ly49s3^{-/+}$ subsets were distinct from the $NKRP1\text{-}B^{dim}$ and $Ly49s3^{+}$ populations because they had lower expression of the immature markers CD62L and CD117 (c-kit) but higher expression of maturity markers such as CD25 and chemokine receptor CX_3CR_1. Furthermore, the functions of $NKRP1\text{-}B^{bright}Ly49s3^{-/+}$ *in vitro* presented characteristics of mature cells, with high production of IFN-γ following activation with IL-12 or IL-18, whereas this secretion was negligible for $NKRP1\text{-}B^{dim}$ and $Ly49s3^{+}$ NK cells. In addition, while $NKRP1\text{-}B^{dim}$ and $Ly49s3^{+}$ presented a high proliferative capacity and survival in culture with IL-2, $NKRP1\text{-}B^{bright}Ly49s3^{-/+}$ subsets died within the first 24 h of culture with low

capacity to proliferate. Taken together, although the NKRP1-B^{bright}Ly49s3$^{-/+}$ subsets were mainly double negative for CD11b and CD27, the authors conclude in relation to their spatial distribution in the body, their phenotypes and their functions that these subpopulations represented the most mature NK cell subtype in rats [5].

Figure 12: Phenotype of (A) human, (B) mouse and (C) rat NK cell subsets.



(A) Human NK cell subsets from blood delineated following their expression of CD56 and CD16, in parent gate CD3$^-$CD14$^-$CD19$^-$ cells. CD16$^+$CD56^{dim} cells represent almost 90 % and CD16$^+$CD56^{bright} cells 10 % of total NK cells. Picture from unpublished results. **(B)** Mouse splenic NK cells, from parental gate on CD3$^-$NK1.1$^+$ cells, representing the main mouse subpopulation of NK cells. Picture from unpublished results. **(C)** Rat NK cells subpopulations from blood gated on CD3$^-$NKRP1A$^+$ cells, distributed following the expression of Ly49s3$^+$ and NKR-P1-B$^+$. Reproduced with permission of the Journal of Immunology, Copyright 2012, in [5].

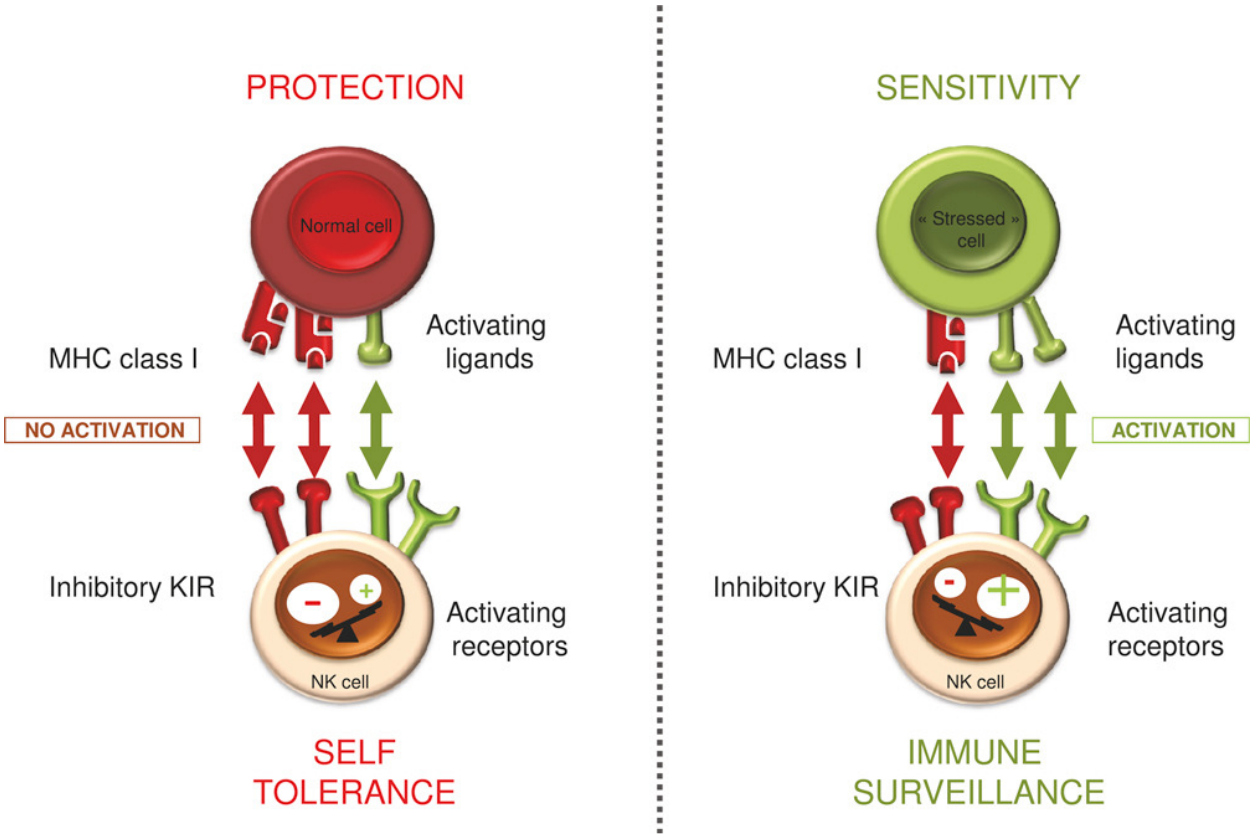
2.8.2. NK cell biology

NK cells are well characterized as important players of innate immunity against infections and neoplasms. Thus, NK cells are quickly recruited to the site of inflammation and can then respond by their cytotoxic functions against aberrant cells and by their secretion of cytokines that support the elaboration of the adaptive immune response [493]. However, NK cells could also down modulate the immune response via direct killing of over activated T cells and secretion of anti-inflammatory molecules, such as IL-10 to limit autoimmune disease [510]. Furthermore, it was recently reported that NK cells could have memory function in particular in the mouse model of CMV infection [511] or following *in vitro* cytokine stimulation [512, 513]. This modulation of NK cell functions may be mediated by two mechanisms including stimulation of NK cells by their inhibitory and activating receptors [514, 515] and by cytokine activation [493, 516].

The capacity of NK cells to recognize and kill modified cells was firstly described by Kärre et al. as the "missing self" recognition, where NK cells become cytotoxic against cells that do not express MHC class I molecules [517, 518]. This established the principle of self-tolerance by NK cells, as MHC class I is normally ubiquitously expressed in the organism, but could be down-regulated during viral infection or cellular transformation [519, 520]. Then the molecular basis of this missing self-tolerance was more extensively investigated following the discovery of inhibitory NK cell receptors with affinity for MHC class I molecules or related molecules, such as killer immunoglobulin receptors (KIR) in human [521, 522] and Ly49 in rodent [523]. The interaction of the inhibitory receptor with its ligand leads to a cascade of intracellular inhibitory

signals in NK cells, mediated by the cytoplasmic tail of these receptors: the immunoreceptor tyrosine-based inhibitory motif (ITIM) [524]. This theory was tested in animal models where the inoculation of MHC class I deficient tumor cell lines resulted in tumoral clearance, while the same cell line presenting MHC class I results in development of the tumor. The authors confirmed that this mechanism was mediated by NK cells as their depletion *in vivo* using anti-asialo GM1 antibody abolished this tumor reduction [517].

Figure 13: Mechanism of NK cells non-self or transformed self-recognition.



NK cells are able to sense its target cells by their large panel of activating and inhibitory receptors. The self-tolerance by NK cells is achieved when inhibitory signal dominate on activating signal, meaning when the density of inhibitory ligand, MHC class I, is more dense than activating ligand on target cell, as is mainly le case for normal cells. However, NK cells become activated then target cells up regulated their activating ligands and/or down modulated their inhibitory one, as it the case in stressed cells. Published in [9](Open access article).

Nowadays we know that the mechanism of NK cell cytotoxicity and secretion is mediated by a fine balance between signals coming from their inhibitory and activating receptors and their integration by NK cells. A simplified vision states that NK cells quantitatively integrate activating and inhibitory signals provided by the target cells to become activated or not (figure 13) [525]. In human, the activating receptors of NK cells, NKG2D, NKp46, NKp30, NKp44, the activating form of KIR, described as KIR-S, and CD16 (or FcγRIIIa receptor), when activated by their cognate ligands trigger cytotoxicity and production of cytokines, through activation of intracellular signaling pathways [526]. Some ligands for these activating receptors remain unknown, nonetheless NKG2D ligands, such as MICA and MICB and NKp30 ligand, as B7H6

or Bat-3, were described to be up regulated in stressed cells leading to NK cell cytotoxicity [527-529]. The activation of NK cells could also be mediated by activation of CD16 by the Fc part of Ab coated to a target cell, a mechanism called ADCC (Figure 10) [530]. However, several mechanisms are developed by tumor cells and viruses to evade NK cells' immune surveillance by up regulating their level of inhibitory ligands [531]. The best interactions characterized so far are: KIR2DL1 and L2/3 molecules with MHC class I type C (HLA-C) and KIR3DL1 with HLA-B with the Bw4 epitope. Another inhibitory receptor well characterized is NKG2A, which recognizes a non-classical MHC class I molecule, called HLA-E [532].

2.8.3. NK cells in CNS and brain tumor

This subsection refers to the PAPER I that extensively reviewed the existing literature about NK cell phenotype and physiology in CNS disorders including brain tumors (PAPER I).

2.8.4. NK cells as a tool for immunotherapy

To date, conventional therapies have failed to cure patients with advanced solid tumors. This failure of traditional approaches prompted the development of immune based cancer therapies. The previous section summarized several approaches developed to treat GBM patients, including adoptive transfer of tumor associated antigen specific CTL and active immunotherapy by vaccination with tumor associated antigen alone or loaded to DC. However, these strategies need the characterization of antigens that are simultaneously highly expressed and specific to the tumor but also enough immunogenic to elicit proper immune response. Unfortunately, up to now most tumor associated antigens do not reach these criteria. Furthermore, many tumors down regulate their expression of MHC class I molecules making them resistant to T cell lysis. In addition several adverse effects appeared in previous clinical trials of adoptive transfer of T cells including vitiligo, uveitis and retinitis [533, 534].

The basis of clinical use of NK cells to treat cancer started in the 80's with utilisation of LAK, that achieved mixed results with variable to poor clinical responses in melanoma patients [535], metastatic renal cell cancer and colorectal cancer [536]. As already discussed several phase I and I/II trials using LAK were tested for glioma patients, although no randomized phase II clinical study was performed so far. Furthermore, the comparison of immunotherapy using IL-2 alone or in combination with LAK did not show statistical difference in a phase III trial for the treatment of patients with advanced renal cell carcinoma [537]. In addition, the cellular composition of LAK cells was often variable with T cells as the dominant cell type that could mediate adverse effects of graft versus host disease [538, 539]. This toxicity was observed in up to 33% of glioma patients following intracranial adoptive transfer of LAK associated to IL-2 [393, 395]. However, further studies on purified NK cells gained better results, with manageable side effects, to treat hematologic and solid cancers including GBM [404, 540]. The recent advance in NK cells biology highlighted the potential of these cells for adoptive immunotherapy against tumors. Thus, in addition to their tumoricidal functions, NK cells are competent to activate potent adaptive immune responses against cancer, but also may present memory like functions [512]. This section will go through the existing methods to get clinical grade NK cells as well as clinical trials based on these cell types.

2.8.4.1. Existing methods for obtaining clinical grade NK cells

Different techniques were developed in order to get high quantities of pure NK cells that satisfy good manufacturing procedures (GMPs) to be safe for adoptive transfer to patients. All these methods were recently reviewed by Sutlu et al. and then by Koepsell et al. [540, 541]. Most frequently, NK cells were isolated and then amplified from PBMCs collected from whole blood or leukapheresis [542-550]. However some teams also used NK cell lines: NK-92 [551-553] or NK cells differentiated from umbilical cord blood [554]. The advantage of using the NK-92 cell line is that these cells do not express inhibitory KIR, making them highly aggressive against many tumors. Furthermore, it simplifies the process of cell preparation for adoptive transfer as this cell line is uniform from batch to batch following culture, with high proliferation rate [551, 555]. Nonetheless, as a tumoral cell line, the use of NK-92 presents the risk that after injection of these cell grafts, the host develops a secondary lymphoma [553]. Nonetheless, usually NK-92 cells are irradiated and so this risk is minimized [552, 553]. Then the technique developed by Spanholtz et al. from umbilical cord blood presents the advantage to be a clinically applicable GMP procedure and to get high amounts of NK cells, with good cytotoxic function and without using feeder cells and animal products [554].

The process of NK cell purification is mainly based on magnetic separation. Here again the protocols found in the literature are heterogeneous. While simple depletion of T cells by targeting CD3 receptor was used [545], the most frequent protocol applied a subsequent CD56-positive selection that confers an increased NK cells purity [545, 546, 550, 556, 557]. Alternatively, negative enrichment of NK cells was also developed, by depletion of $CD19^+$ B cells, $CD33^+$ myeloid cells and $CD4^+$ T cells [548].

The following steps of NK cell expansion use a mixture of cytokines, generally IL-2 alone [545] or associated with IL-15 [546], but many protocols include irradiated feeder cells, such as PBMCs [543, 546, 548, 549] or human cell lines [404], EBV transformed cells [544] as well as engineered leukemic cell lines [547, 558]. All these methods use clinical grade serum free culture medium, such as X-VIVO™ serum-free media (BioWhittaker, Vervier, Belgium), AIM V™ (Life Technologies, Grand Island, NY) or stem cell growth medium (CellGenix, Freiburg, Germany), supplemented with human AB serum or certified fetal bovine serum [545, 549, 559]. These large numbers of protocols using different cellular sources, enrichment and culture methods to get clinical grade NK cells make the comparison of their efficiency difficult. However, in the future when more NK cell based clinical trials will be completed, this difference in terms of purity and efficiency may help to delineate the optimal and reproducible methods to achieve safe immunotherapy.

2.8.4.2. NK cell based clinical trials

2.8.4.2.1. Adoptive transfer of autologous NK cells

Numerous preclinical data on animal models strongly suggested the amenability of adoptive transfer of autologous NK cells for cancer patients. In particular in NOD/SCID mice where the adoptive transfer of activated NK cells from acute myeloid leukemia (AML) patients demonstrated *in vivo* cytotoxicity against autologous AML blasts [560]. Several cancer types have been targeted using ex vivo activated NK cells in human, including metastatic renal cell carcinoma [561], recurrent glioma [404], metastatic breast cancer [562]. These studies demonstrated a positive clinical response of patients to the NK cell based therapy. However, in other studies on breast cancer and lymphoma patients, although the results reported the

feasibility and the safety of this therapy, no clear clinical efficiency was demonstrated [563, 564]. Nonetheless, these studies revealed that the treatment was able to increase the NK cell counts in the blood of patients [563, 564], as well as their ex vivo cytotoxicity against tumor cell lines [565]. The reason for the discrepancy in efficiency following these studies is still enigmatic, as many of them did not perform a detailed phenotyping of NK cells before infusion, to be able to delineate the level of their activation and their expression of inhibitory *versus* activating receptors. Nonetheless, the NK cells activation time *ex vivo* could have an influence, as previously shown by Alici et al. [566]. Thus, with myeloma patients, long term *ex vivo* activation of NK cells favoured cytotoxic capacity of NK cells against autologous tumor cells [566]. The disadvantage of using autologous cells for NK cell preparations is that in many cases cancer patients presented low PBMC counts and also impaired immune function [567]. Furthermore, many cancer cells aberrantly over express inhibitory ligands for self NK cells as a mechanism of immune escape; this renders the adoptive transfer less efficient [531]. This is why much work focuses now on the use of allogeneic cells, especially because of encouraging clinical results as the next section will explain.

2.8.4.2.2. Adoptive transfer of allogeneic NK cells

The retrospective studies on hematopoietic stem cell (HSC) transplantation, as well as on clinical trials to treat cancer highlight the potential of using allogeneic NK cells for adoptive transfer therapy [568-570]. As demonstrated by Igarashi et al., the grafted NK cells cytotoxicity *versus* tumoral cells was maximal when KIR and MHC class I was mismatched between donor and recipient in the context of melanoma and renal cell carcinoma [571]. In other words, when recipients do not express one or more KIR ligands expressed by the donor [568, 571]. Indeed, the feasibility of allogeneic NK cell based therapy was proven by the complete remission of AML patients following haploidentical (HLA-mismatched) adoptive transfer of NK cells [569]. The same promising results were obtained in a pilot study including children with AML where all patients engrafted with allogeneic NK cells following an immunosuppressive regimen remained in remission with a median follow-up time of 964 days [556]. These allogeneic NK cell infusions were well tolerated without signs of graft versus host diseases [556, 569]. This was also demonstrated in pediatric patients with acute lymphoblastic leukemia (ALL), AML and chronic myeloid leukemia (CML) [572, 573]. Then, Shi et al. [574] confirmed the *in vivo* expansion of infused NK cells in patients with multiple myeloma previously observed by Miller et al. in AML [569]. The study of Brand et al. revealed that adoptively transferred radiolabelled allogeneic NK cells could be detectable in whole body up to 6 days by scintigraphy [575]. Moreover, accumulation of NK cells was detectable in 50 % of evaluable metastases of renal cell carcinoma [575]. Another allogeneic approach using NK-92 to treat advanced renal cell carcinoma and melanoma, indicated that it could be safe with potential benefit for the patients [552, 553].

In summary, all preclinical and clinical evidence suggests that allogeneic and autologous infusion of NK cells could be amenable in a variety of cancers. Nonetheless, up to now this therapy is limited to a low number of patients, in relation to the lack of a large scale clinical grade NK cell expansion protocol. Much more studies are needed to delineate the optimal NK cell therapy scheme to be applicable for different types of cancer individually.

3. AIMS OF THE STUDY

The review of the literature revealed the lack of knowledge about the implication of NK cells in normal brain physiology. Up to now, the majority of information pertaining to brain NK cells has been obtained from CNS associated pathological circumstances.

PAPER I: The goal of this paper was to:

a) investigate the occurrence and the phenotypes of NK cells from brain of naïve mice as well as from CSF of non-pathological brain (NPB) patients
b) discuss these new findings in the context of existing knowledge related to CNS NK cells.

Although our understanding of the brain tumor microenvironment has improved the last 2 decades, there is still a great need for consolidated knowledge of the particular immunological status of GBM patients, in the periphery as well as within the tumor bed.

PAPER II: This paper aimed to:

a) elucidate the overall immunological status of GBM patients in the tumor microenvironment relative to the periphery compared to healthy age matched controls,
b) unravel the integrated inflammatory interplay between the GBM and immune cells
c) determine the impact of increased infiltration and activation phenotypes of T cells on patients' survival outcomes
d) identify possible novel therapeutic targets for immunotherapy.

Indeed, current treatment has failed to cure GBM patients, so there is an urgent need for the development of new therapeutic strategies and targets. As previously reviewed many immunotherapies are in development to treat GBM patients. However the use of NK cells is still marginal despite this being a promising approach to treat cancer. In regard of the implication of NG2/*CSPG4* in GBM aggressiveness we investigated a new combination immunotherapy using specific mAb9.2.27 against NG2/*CSPG4* and NK cells in preclinical animal models. Thus, pure "untouched" rat NK cells were required, as we planned to use nude rats bearing human GBM cells as preclinical models for testing our combination immunotherapy.

PAPER III: This methodological paper aimed to:

a) design an efficient purification method to obtain highly pure "untouched" rat NK cells as no commercially available kit exists to purify untouched rat NK cells
b) determine whether "untouched" NK cells differ in phenotypes and activation profiles from positively selected NK cells using anti-NKRP1A mAb.

PAPER IV: This last paper aimed to

a) investigate the therapeutic efficacy of the combination mAb9.2.27 against NG2/*CSPG4* and activated NK cells against several GBM models
b) identify the molecular and cellular mechanisms of therapeutic efficacy.

4. SUMMARY OF RESULTS

PAPER I: Natural Killer Cells in Central Nervous System Disorders.

This first paper is a brief review about the implication and modification of NK cells in periphery as well as in the brain in the context of CNS disorders. This work firstly critically reviewed the literature about NK cell phenotype and function in peripheral blood of patients with brain tumors, infections, neurodegenerative diseases, acute vascular and traumatic damages as well as mental disorders. Then, the second part of this review provides original experimental data describing the presence of NK cells within CNS of naïve C57BL/6 mice. For the first time we demonstrated the presence of $CD3^{-}NK1.1^{+}$ NK cells with $CD11b^{low}CD27^{+}$ phenotypes, characteristic of immature cells in the brain of mice. Then we also confirmed previous observations from Hamann et al. showing that CSF of non-pathological human brain presented NK cells with the $CD56^{bright}$ phenotype [576]. Then we further discussed these observations in relation to the existing literature on brain infiltrated NK cells in context of CNS disorders. We observed that the literature in relation to NK cell physiology in CNS disorders is really sparse. Moreover, when information is available, it is difficult to conclude as often NK cell characterization is not well performed, due to single staining of CD56 or CD57 that could also recognize T cells. However, it appeared that infiltration of NK cells following CNS disorders could have both neuroprotective and neurodegenerative implications. These paradoxical functions of NK cells could be mediated by their cytokine secretion as well as their cytotoxic functions that govern their cross talk with CNS resident cells, such as microglia and T cells. This review delineates the importance of future efforts to determine if NK cells are a cause or a consequence of brain disorders, as for neurodegenerative diseases and mental disorders. Indeed, a better comprehension of NK cells in brain pathogenesis might help to delineate new therapeutic targets for the treatment of CNS disorders.

PAPER II: Elevated CD3+ and CD8+ tumor–infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor and peripheral microenvironment.

In this second paper we studied the impact of the infiltration of $CD3^{+}$ T cells in the tumor biopsy, as well as $CD4^{+}$ and $CD8^{+}$ cells on GBM patients' survival. These analyses were done by single immunohistochemistry staining on 65 GBM patient biopsies. These results revealed a significant positive correlation between the infiltration of $CD3^{+}$ and $CD8^{+}$ cells and the patients' survival in multivariate analyses, while no correlation with $CD4^{+}$ cells was observed.

Then we investigated the phenotypes of T cells from PBMC and GBM biopsies of 8 patients versus phenotypes of T cells from PBMCs of 9 healthy gender and age matched donors, using multi-parametric flow cytometry. We observed a diminution of the proportion of T helper cells, $CD3^{+}CD4^{+}$, in PBMC and biopsy of patients compared to controls. However no difference of cytotoxic T lymphocytes (CTL), $CD3^{+}CD8^{+}$, were identified. Then, we examined in detail the phenotypes of the T cell subpopulations. We observed several modifications on CTL phenotypes following their infiltration in GBM biopsy, including a diminution of their expression of co-stimulatory molecules, CD28 as well as a diminution of adhesion molecules CD56 and CD62L. In addition all T cells lacked the expression of co-stimulatory CD27 and CD154 molecules in GBM biopsy. Furthermore, while we did not observe differences in the presence of natural

occurring T_{regs}, $CD3^+CD4^+Foxp3^+$, much more T helper cells expressed CTLA-4 in blood of patients compared to controls. Although no natural occurring T_{regs} were detectable in GBM patient biopsies, we identified a novel T_{regs} population, $CD3^+CD8^+CD28^-Foxp3^+$, in 60 % of analyzed patient biopsies, but not in their blood. Additionally, we observed that approximately 2 % NK cells infiltrated the GBM biopsies and presented mainly $CD56^{dim}CD16^+$ phenotypes with more than 50 % expressing the activating receptor NKG2D. Some NKT cells were also detected; however the presence of B cells was really low.

The analyses of antigen presenting cells (APC), mainly macrophage/microglia, gated by size *versus* granularity (on FSC *vs* SSC, by flow cytometry) indicated that this cell type represented the major subpopulation infiltrating the GBM biopsy. These cells were almost negative for costimulatory molecules, CD40, CD80 and CD86. However, 50 % expressed NKG2D activating receptor, but also expressed inhibitory receptors ILT_2, ILT_3 and ILT_4. Interestingly, a third of them expressed CD4 and CD8 co-receptors.

To elucidate how the tumor cells interact and respond to the immune cells, we further investigated the receptor/ligand phenotypes of tumor cells, operationally defined as $CD45^-CD31^-$ cells. Contrary to several published reports, the majority highly expressed MHC class I molecules (HLA-A,B,C), but were consummately negative for non-classical MHC class I molecules, such as HLA-E and HLA-G. The tumor cells did not express CD70, Fas and MICB, but were positive for MICA and Fas-L. Interestingly, while 30 % of neoplastic cells expressed the ectonucleotidase CD73, $CD39^+$ cells were almost inexistent, which appeared to be the inverse on APC, where the majority was $CD73^-$ but some presented CD39 at their cell surface.

All together this paper revealed an integrated anti-inflammatory interplay between the GBM and immune cells. Indeed, while peripheral CTL of patients presented normal phenotypes, the GBM infiltrated T cells down regulated their molecules of activation. Furthermore, GBM attracted $T_{regs,}$ $CD8^+CD28^-Foxp3^+$ that may play a role in the anti-inflammatory environment mediated by the tumor but also can immunotolerize GBM resident APC. Furthermore, T helper cells appeared to be down modulated by their high expression of inhibitory CTLA-4 molecules. This is further amplified by the presence of the ectonucleotidases CD73 and CD39 well known for their potential of NK cells and T cells inhibition by generation of extracellular adenosine mobilization.

Nonetheless these findings indicated potential for immunomodulatory therapies for GBM management. Thus, the correlation of increased T cell infiltration with improved patient survival implies potential for immunomodulatory therapies for GBM management. Potential immunotherapy strategies might include allogeneic NK cell transfer, as well as targeting the $CD8^+CD28^-Foxp3^+$ Tregs subpopulation and CTLA-4 inhibitory receptor

PAPER III: Novel method for isolating untouched rat natural killer cells with higher purity compared with positive selection and fluorescence-activated cell sorting.

This is a methodological paper describing the protocol to get highly pure untouched NK cells from rats. We set up a novel cocktail of biotin mAb to get rid of non-NK cells. This included mAb anti- CD5, -CD45RA, -CD172a, -endothelium, -erythrocytes and –granulocytes, in order to deplete T cells, B cells, monocytes/macrophages, cells from endothelium, erythrocytes and granulocytes, respectively. We took advantage of the Miltenyi Biotec B.V

magnetic separation technology, by catching the primary biotinylated mAb with a mAb anti-biotin coupled to magnetic microbeads. The separation of NK cells from non-NK cells was then done by applying the stained cell suspension through a magnetic field. By this way, we were able to purify NK cells from LEWIS, Fischer and athymic nude rats with a purity of respectively 97.7± 0.7 %, 96.6± 0.8 % and 88.3± 1.5 %.

Up until now, the majority of research teams working with rat NK cells used positive selection by magnetic separation. We further compared the integrity of purified NK cells following the 2 purification methods. We observed that positive selection modified NK cells physiology by increasing their proliferation rate, survival as well as their cytotoxicity and cytokine secretion capacity *in vitro*. This indicated that negative purification methods should be privileged for fundamental studies on rat NK cell biology as it presents the advantage to be free of undesired method related activation.

PAPER IV: Targeting glioblastoma with NK-cells and mAb9.2.27 against NG2/CSPG4 diminishes tumor progression and prolongs animal survival.

In this last paper we investigated our novel treatment using mAb9.2.27 against NG2/*CSPG4* in combination with activated NK cells. We applied our treatment in 3 different cohorts of athymic nude rats bearing intracranial human GBM. The first cohort was implanted with human GBM cell lines U87MG, presenting > 90 % antigen positive cells recognized by the mAb9.2.27. The second cohort was implanted with U251-NG2 GBM cells that express rat NG2/*CSPG4* that is poorly recognized by the human NG2/*CSPG4* specific mAb9.2.27 resulting in reduced affinity. These GBM cells were only recognized as 30 % antigen positive by mAb9.2.27. The final cohort bearing GBM patient biopsy, P3-30 > 70 % antigen positive, with a good affinity for the mAb9.2.27 and a more aggressive disease course compared to previous cell line based tumors. The survival of animals bearing GBM treated by our combined treatment presented a significant prolonged survival compared with the controls, and monotherapy groups, for both U87MG and U251-NG2 (P3-30 cohort survival is under investigation for the paper revision). This increased survival was more pronounced on U87MG cohort, where 60 % of rats presented tumor remission on MRI and histology following combination treatment. The increased survival was associated with elevated cellular apoptosis in the tumor bed, demonstrated by tunnel assay, as well as diminished proliferation, demonstrated by a reduction of Ki67 labeling index. Furthermore, we observed by immunohistochemistry a massive recruitment of cells expressing myeloperoxidase activity through the choroid plexus. In addition, much more cells were positive for Granzyme B, IFN-γ, CD8 and ED1 following combination treatment compared to controls. As macrophage/microglia could express all these molecules we further launched the P3-30 cohort in order to test our treatment following the systemic depletion of macrophages using clodronate. Interestingly, although combined NK+mAb9.2.27 treatment diminished tumor growth associated with attenuated proliferation and increased apoptosis, the macrophage depletion abolished these therapeutic effects, as well as the recruitment of ED1 and CD8 positive cells. By flow cytometry staining on brain lysate, we further confirmed the diminution of recruited macrophages 3 days following the clodronate treatment, while the microglia population was untouched. Furthermore, we characterized the phenotypes of the recruited macrophages, in the successful combination treatment, and could demonstrate diminished CCR2 expression and that microglia had increased expression of MHC class II molecules as well as ED1, but diminished ED2 expression.

We further investigated the capacity of mAb9.2.27 to modulate the microglia and NK cell cytotoxic functions *in vitro*. We were surprised to observe that while microglia could significantly increase their cytotoxic capacity against GBM cells with mAb9.2.27, NK cells could not. Furthermore, mAb9.2.27 as well as IFN-γ, were able to attenuate the tumor promoting effect of tumor associated microglia/macrophage sorted from human GBM biopsy or *ex vivo* microglia cultured with GBM supernatant. In addition, we observed that following co-culture of NK cells with GBM cell lines, these cells secreted IFN-γ and TNF-α.

In conclusion, we demonstrate that NK+mAb9.2.27 may represent a potent therapeutic strategy for treatment resistant NG2/*CSPG4* expressing GBMs. Nonetheless, future studies must examine the safety of this therapy, to validate risk *versus* therapeutic benefit of such therapy to treat GBM patients.

5. DISCUSSION

5.1. Particular experimental design

5.1.1. Relevance of an *in vivo* model for preclinical validation of NK+mAb9.2.27 to treat GBM

We decided to validate our novel combination treatment on rats xenografted with human GBM for several reasons. First, the rat's brain presented the advantage of its larger size compared to mouse brain [577]. This allows a more precise stereotactic tumor spheroid implantation. Furthermore, following the GBM engraftment in rat brain, it results in a bigger tumor mass than in mice that presents similar challenges of bio-distribution of therapeutic compounds as in the human situation. Rats present also a longer period prior to death allowing for evaluation of therapeutic efficacy [577]. This larger tumor mass was also attractive for the rationale of delivery of the mAb9.2.27 by convection enhanced delivery in association with NK cell suspension injection, that could be done directly in the tumor core [577] without affecting the volumetric balance. Moreover, the rat is genetically closer to human, making rat's brain more representative of human brain [578]. The literature pertaining to *in vivo* study of rats bearing brain tumors is more comprehensive than for mice [577]. In general the best model to study tumor should recapitulate patients genetic and histologic hallmarks, in particular infiltrative growth and angiogenesis in context of GBM [577, 579, 580].

For our treatment validation we used 3 xenografts models of human GBM in athymic nude rats, with differential aggressiveness. The 2 first models involved the implantation of GBM cell lines, originally derived from patient biopsy, U87MG and U251-NG2. These cell lines were long-term *in vitro* passaged and are by consequence more homogenous than the original patient biopsy. Moreover, they present a circumscribed growth pattern around the injection site *in vivo*, with poor invasion into the parenchyma. Although, these models do not recapitulate all patient characteristics they are well reproducible with respect to the tumor take, growth, angiogenesis as well as survival characteristics [579, 580] and are as such, useful for hypothesis generation, mechanistic studies and initial screening of treatment strategies. We took the advantage of the differential recognition of U87MG and U251-NG2 by mAb9.2.27 in order to first demonstrate the specificity of our treatment and then to emphasize the importance for the future clinical application to selectively enroll patients with high antigen expressing biopsies for high affinity binding mAb9.2.27. As described in the paper IV, *in vivo* results presented beneficial outcome on animals treated with our combined immunotherapy, with 60 % of tumor remission in U87MG treated animals. As these GBM cell lines xenograft models are considered to be easier to treat compared to more aggressive models, we further decided to complete and reinforce our observations using a more aggressive model, such as xenograft models of patient GBM biopsy, P3-30. Recently, our team validated this brain tumor model as highly relevant for the investigation of new therapies, due to its cellular heterogeneity, invasive and angiogenic phenotype that closely resembles that of GBM patients [73, 581].

On the other hand, it should be noted that some divergence of the phenotypes from immune infiltrated cells could be denoted between rodent and human. Indeed, we characterized that there were more macrophage-like cells ($CD11b^{+}CD45^{high}$) compared to microglia ($CD11b^{+}CD45^{low}$) in newly diagnosed patient GBM biopsies (PAPER II), as also shown by other

research groups [582]. However, in rodent models of glioma, there was a predominance of microglia instead macrophages (PAPER IV) and [165].

We decided to work on the xenograft models of immunodeficient rat bearing human GBM because our mAb9.2.27 is highly specific for the human form of NG2/*CSPG4* proteoglycan. By consequence, we avoided the use of syngeneic rat model where our mAb9.2.27 would have inefficiently recognized rat NG2/*CSPG4* on neoplastic cells. Furthermore, the implantation of human GBM in immune-competent rat results in tumor rejection. In conclusion, our results do not take in consideration the possible influence of regulatory T cells that could diminish the treatment efficiency.

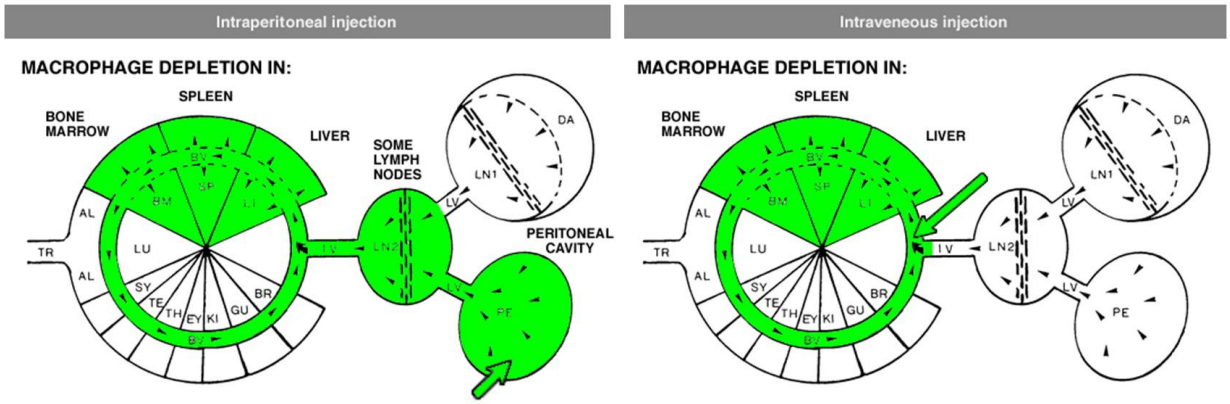
Finally, we decided to perform the intracranial adoptive transfer of rat NK cells, in order to investigate whether NK cells influence and/or cross talk with the host cells. Nonetheless, future studies should use human NK cells to further delineate their real capacity to kill human GBM.

5.1.2. Relevance of macrophage depletion using clodronate

Clodronate (dichloromethylene diphosphonate) is a member of bisphosphonates family developed by Roche Diagnostic laboratory for the treatment of osteolytic bone diseases and osteoporosis. It has been characterized for its capacity to block osteoclast functions as well as its apoptosis promoting effect [583]. Later it was discovered that clodronate encapsulated in liposome had the ability to induce macrophage apoptosis following their phagocytosis *in vivo* and *in vitro* [584, 585]. The clodronate and liposomes are not cytotoxic *per se* for macrophages. Indeed, free clodronate does not pass by the cellular membrane of macrophages. However, clodronate incorporation in liposomes leads to the engulfment through phagocytosis by macrophages. Intracellular liberation of this drug, following liposome disruption by the lysosomal phospholipases, further mediated cellular apoptosis by competing with ATP-binding [584, 586, 587]. This method does not induce the secretion by dying macrophages of pro-inflammatory mediators, such as nitric oxide (NO) [588]. Furthermore it was shown to selectively induce apoptosis of macrophages [587] and phagocytic dendritic cells [589]. Thus, several work proved no modification on lymphocyte and neutrophil *in vivo* following liposome clodronate (LC) injection [586, 587, 590, 591].

The route of LC administration was shown to determine the body compartment from which macrophages will be depleted [584, 587]. In our case, we preferred intraperitoneal (IP) injection to intravenous injection (IV). Indeed, while IP and IV injection were shown to deplete macrophages from bone marrow, spleen, liver and blood venous circulation, IP depleted in addition macrophages from intraperitoneal cavity as well as from some lymph nodes [587, 592](Figure 13). Moreover, a large volume of LC should be administered (1 ml/ 100 gram animal body weight), but current guidelines for maximal injection volumes in rat recommend intraperitoneal injection for volume superior to 0.5 ml. It appeared therefore safer to inject LC in the large cavity that constitutes the peritoneum compared to intravenous route to limit injection-related adverse reactions [593].

Figure 13: Route of LC injection determined the body macrophages depletion.



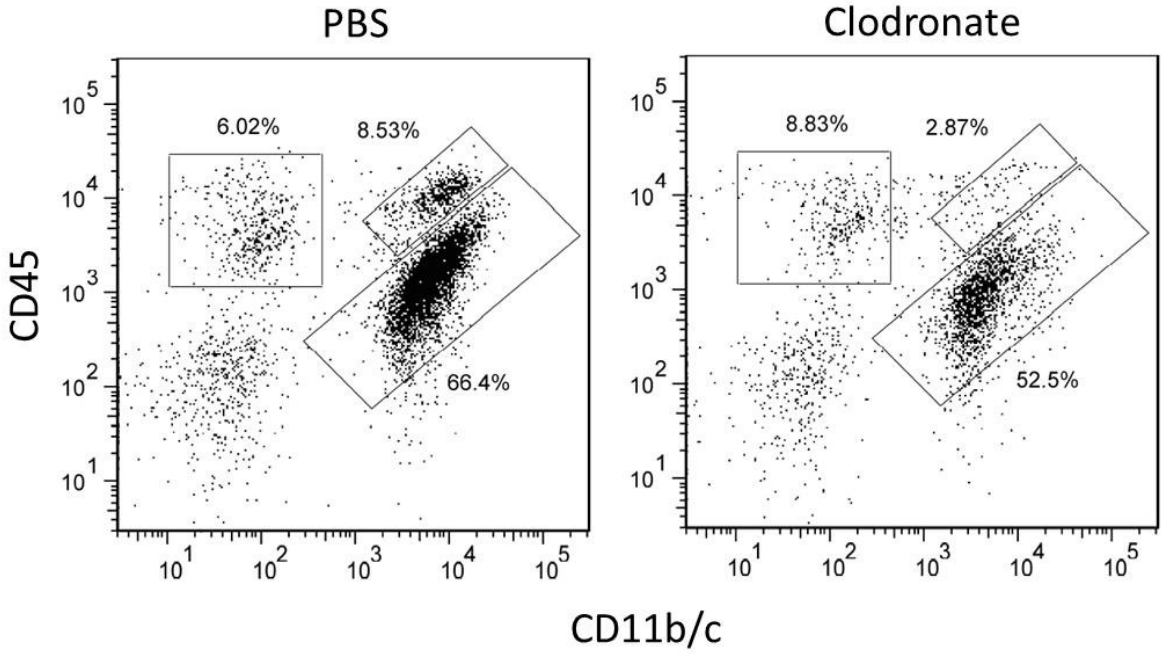
Comparison of macrophage depletion by intraperitoneal or intravenous injection of LC. Both methods depleted macrophages from bone marrow, spleen, liver and blood venous circulation and intraperitoneal injection further depletes macrophages from the peritoneal cavity and some lymph nodes. Reproduced with the permission of Dr. Nico van Rooiien.

We hypothesized that the beneficial outcome in the rats bearing GBM cell lines U87MG and U251-NG2 was in relation with the massive recruitment of pro-inflammatory macrophages following the treatment (PAPER IV). To prove this hypothesis, we embarked on systemic macrophages depletion using LC injection in the more aggressive P3-30 models, to diminish brain macrophages recruitment following the treatment. In order to keep the population of macrophages as low as possible in the rats following the treatment, we performed one IP injection of LC per week for 4 weeks. While macrophages could be eliminated from spleen within 24 h following LC injection their replenishment may take up to 10 days [586]. However, the recirculation from bone marrow to the blood of macrophages precursor could be quickly achieved within 24 to 48 h [591]. However, we confirmed by flow cytometry 3 days post LC injection that the depletion of macrophages expressing ED2 and ED1 was effective in spleen (PAPER IV). It should be noted that the IP injection of clodronate could not deplete microglia. It was demonstrated by several groups that this could only be achieved by LC intracranial administration [594, 595]. Indeed, we observed that we were able to decrease drastically the macrophages recruited to the brain without major modification of microglia population (Figure 14). Furthermore, it is possible that the remaining macrophages in the CNS following CL injection are the resident CNS-associated macrophage commonly found in the normal brain.

In addition, we did the first clodronate injection the same day of the combination NK+mAb9.2.27 treatment. Indeed, a depletion of macrophages earlier in the protocol, such as some days following the tumor implantation could have resulted in an impaired tumor growth by reducing the recruitment of tumor associated macrophages with typical M2, anti-inflammatory phenotypes known to promote tumor growth [139, 155]. Thus, as it was shown in xenograft mouse model of melanoma [596], diffuse malignant mesothelioma [597] or orthotopic rat prostate tumor model [598] the injection of clodronate before the complete *in vivo* tumor engraftment diminished tumor volume and increased animal survival compared to non-depleted animals. Indeed, in organotypic brain slice culture *ex vivo* ablation of microglia using LC was able to reduce tumor invasion [599]. Furthermore, the concomitant *in vivo* glioma inoculation to the depletion of brain macrophage/microglia in mice resulted in a smaller tumor mass compared to controls [600]. However, in our models the tumor was already well established before

macrophage depletion and we did not observe tumor reduction in this macrophages depleted group (PAPER IV). In contrary, this macrophage depletion abolished the therapeutic benefit mediated by NK+mAb9.2.27 treatment, indicating that these cells are implicated in the mechanism leading to tumor reduction.

Figure 14: Depletion of macrophages infiltrating the brain 3 days following LC injection.



Comparison of cellular composition of resident lymphocytes, $CD45^{high}CD11b/c^{-}$, macrophages, $CD45^{high}CD11b/c^{+}$ and microglia $CD45^{low}CD11b/c^{+}$, 3 days post IP injection of LC.

Nevertheless, we cannot exclude that dendritic cells do not have also an impact on animal survival, as LC depleted both macrophages and dendritic cells. We did not use anti-OX-62 (CD103) mAb, in our flow cytometry staining, to be able to differentiate MHC class II positive activated macrophages from dendritic cells. Indeed, both these cell types share the expression of MCH class II and only OX-62 could distinguish them from dendritic cells [601-603].

5.2. Implication of experimental findings

The characterization of NK cells within the naïve brain might lead to the interrogation of the nature and/or role of NK immune surveillance and possible emergence of brain tumor, in the context of immune surveillance by both NK cells (PAPER I), but also cytotoxic T cells [111]. 10-15 % of human cancers worldwide were shown to be the consequence of virus infection [604, 605]. As already discussed in the introduction (Section 3.6.1.15), several research teams demonstrated that 90 % of patient GBM biopsies presented CMV infection [356]. The mechanism of glioma promotion following viral infection involves direct oncogenes, such as IE1 expression in GBM cells which contributes to GBM proliferation [354] and indirect carcinogens that facilitate GBM expansion via mechanism of immune cell evasion [606, 607]. It was characterized that CMV infected cells evade T cells' immune response by modulation of proteins

such as US2, US3, US6, and US11 that limit antigen presentation and the expression of MHC class I molecules [608]. Nonetheless, they do not become more susceptible to NK cell lysis as in addition, they express other viral products such as UL16, UL18 and UL40 that interfere with expression of activating or inhibitory cognate ligands [606].

On the other hand, many authors characterized the *in vitro* resistance of GBM cell lines to resting NK cell lysis, mainly in relation to their high expression of MHC class I molecules [226, 403], and non classical molecules, like HLA-E [609]. Our results also reported the expression of classical MHC class I molecules on patient biopsies, while we did not corroborate the expression of HLA-E (PAPER II). However, while resting NK cells were not able to kill GBM cells *in vitro,* it seems that these tumor cells were less resistant to autologous and allogeneic IL-2 activated NK cells, despite their expression of MHC class I molecules and HLA-E [400]. Indeed, Castriconi et al. demonstrated that this cytotoxicity is related to the high expression of NKp46, NKG2D and DNAM-1 ligands on GBM cells as their simultaneous blocking abrogated the NK cell lysis [400]. This susceptibility of GBM cells indicated activated NK cells as a promising candidate for adoptive cellular therapy for GBM. Furthermore, these results suggested that the pre-activation of NK cells *in vivo* is a prerogative for a successful immunotherapy. Future *in vitro* NK cell expansion/activation methods should provide cells with high level expression and diversity of activating receptors.

Nonetheless, the high immunosuppressive environment created by the tumor should be taken into consideration for future NK cell adoptive transfers. Indeed, we observed the presence of T_{regs} cells and macrophage/microglia with anti-inflammatory phenotypes that may block efficiency of NK cell based therapy (PAPER II). This is why this treatment should be conceivable following the surgical resection, when the maximum of the tumor mass is removed, and by consequence immunosuppressive factors are less prominent.

The results of the PAPER IV indicated that implantation of NK cells expressing pro-inflammatory molecules could be important for the recruitment and the modulation of pro-inflammatory macrophage/microglia, that further become competent for neoplastic cells destruction. Indeed, we observed that the beneficial outcome is ultimately correlated to the recruitment of systemic macrophages through the brain as clodronate treatment abolished this effect. Furthermore, we observed the presence of microglia with more pro-inflammatory phenotypes, indicated by higher expression of MHC class II molecules and ED1. Nevertheless, as microglia can up-regulate their level of CD45 expression in pathological contexts [610, 611] and macrophages may down-regulate it [612], this may complicate the correct distinction of the origins for both these cell types. This is why many authors, including ourselves, rather prefer to denote collectively macrophage/microglia in pathological contexture, including in glioma [164](PAPER II)(PAPERIII).

In addition, we have shown that NK cells alone were not able to prolong animal survival. Indeed, the reductions of tumor size associated with the increased survival were only possible following co-administration with mAb9.2.27. The recruitment of pro-inflammatory macrophages/microglia as well as the presence of pro-inflammatory molecules in the tumor core were maximal following the treatment of NK cells combined with mAb9.2.27. In relation to this experimental evidence we hypothesized that NK cells were the limiting factors to reverse modulate the anti-tumor environment created by the tumor to a more inflammatory one. This further favors the GBM killing capacity of macrophage/microglia using mAb9.2.27. In parallel,

we demonstrated that mAb9.2.27 can inhibit the capacity of tumor associated macrophage/microglia to promote tumor growth. This last property of mAb9.2.27 appears highly promising to diminish the impact of tumor associated macrophage/microglia on tumor expansion. Indeed, several studies characterized these cells as an important target for future clinical application [613]. Furthermore, the impact of mAb9.2.27 on the capacity of these cells to promote tumor neovascularization needs further investigation. Thus we observed by MRI a diminution of contrast agent in animals treated with mAb9.2.27 (PAPERIV). However we did not delineate the mechanism leading to this diminution. This could be a direct inhibitory effect of the mAb on NG2/CSPG*4* positive cells leading to the formation of new blood vessels, as well as the diminution of VEGF secretion by tumor associated macrophage/microglia following cross linking of the mAb9.2.27. Future investigations should be made to clarify this point.

6. CONCLUDING REMARKS

This thesis work leads to multiple new fundamental findings that increase the knowledge of brain immunology in normal and in GBM contexture. The first contribution is the demonstration of the presence of NK cells within the naïve brain in mice and humans. The NK cells presented particular phenotypes generally observed in lymph nodes that are $CD11b^{low}CD27^{+}$ in mice and $CD56^{bright}$ in humans (PAPER I). The other fundamental new finding is the presence of T_{regs} cells with $CD8^{+}CD28^{-}Foxp3^{+}$ in the GBM biopsy (PAPER II). This thesis also corroborates previous observations of the immunological status of GBM patients, such as their general immune suppression. Furthermore, we confirmed the prognostic value of infiltration of T cells in the brain tumor, where a positive correlation of increased patient survival with the degree of brain infiltrated $CD3^{+}$ T cells was found. The methodological paper of this thesis describes a novel technique to purify pure untouched rat NK cells. This method will be useful for researchers in future studies utilizing pure rat NK cells in large scale studies, as it is a quick, efficacious and relatively cheap method compared to FACS cell sorting. Finally PAPER IV revealed the efficacy of the combined treatment using activated NK cells and mAb9.2.27 to diminish tumor growth in rat models of GBM. This method needs further preclinical validation for future clinical application, but it appears that engineering NK cells for the treatment of GBM presented a promising perspective.

7. FUTUR PERSPECTIVES

In relation to the new findings presented in this thesis several future research perspectives could be delineated. In relation to PAPER I that characterized the presence of immature NK cells in the non-pathological brain, more fundamental studies are needed to further understand their implication and their function in CNS. Is this brain NK cell a specific subset of resident immune cells, as CNS-associated macrophages or are NK cells trafficking to the brain to undergo immune surveillance as already observed with memory T cells? This intriguing question could be investigated further using a large set of experiments. Deeper investigation of their phenotypes may help to delineate the molecules leading to their recruitment to the CNS, but also the function of NK cells within the brain. It could be really informative to further investigate their expression of activating and inhibitory receptors. Then *ex vivo* functional analysis such as cytotoxicity capacity as well cytokines secretion could help to understand their function. Furthermore, in relation to the particular phenotypes borne by brain NK cells, it could be interesting to delineate if particular brain factors could modulate systemic NK cells, by testing *ex vivo* systemic NK cell phenotypes and functions in presence of CSF. The better comprehension of CNS NK cell physiology could be further helpful to delineate NK cell based therapy in a context of brain disorders. Indeed, in the second paper of this thesis we observed the presence of NK cells in the GBM patients' biopsies (PAPER II). Thus, a better comprehension of NK cells in the naïve brain may help to delineate the modification leading to their inability to reject the emerging brain tumor.

In relation to this PAPER II, future investigations should first complete the investigation on NK cell phenotypes and functions. Much more activating and inhibitory receptor should be investigated in these GBM infiltrating brain, and in parallel their ligands should be characterized in GBM, as for example NKp30 and NKp40 receptor and ligands. In general this paper needs further investigation on the functional capacities of characterized infiltrating cells, especially $CD3^+CD8^+CD28^-Foxp3^+$ regulatory T cells that we were the first to characterize in GBM contexture.

In regard of the clinical validation of the combined immunotherapy to treat GBM patients using activated NK cells in association to mAb9.2.27, further clinical evidence is needed for translation to human. In first we should delineate the best way to obtain highly competent NK cells to target human GBM. We should delineate the advantage of using allogeneic NK cells with KIR-mismatch for GBM ligand. Then, it appears essential to characterize the best way of *ex vivo* expansion to obtain highly aggressive NK cells against GBM. Moreover, future toxicological investigation should be designed in order to characterize the safety of our treatment. Finally, it would be highly interesting to further investigate the therapeutic potential of the direct effect of the mAb9.2.27 on the GBM neovascularization. Is mAb9.2.27 able to inhibit the angiogenic function of NG2/*CSPG4* by itself, or does it require the ligation by cells presenting Fc receptors, such as macrophage/microglia? Indeed, further studies to reply to this particular question on the implication of our mAb9.2.27, in association to toxic studies will help for the design and the development of a secure and efficient treatment for GBM patients.

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