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Role of miR-21 in the regulation of microglia immune response to glioma

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Maria da Conceição Monteiro Pedroso de Lima (Universidade de Coimbra) e da Doutora Ana Luísa Colaço Cardoso (Universidade de Coimbra)

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Ao meu Avô Emílio

que a morte levou sem

sem que me visse formado

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Agradecimentos

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Abbreviations

Abbreviations

3' UTR – *3' Untranslated Region*
7-AAD – *7-Aminoactinomycin D*

A

ADP – *Adenosine 5' diphosphate*
AID – *Activation-induced cytidine deaminase*
AIM – *Absent in melanoma 2*
AGO – *Argonaute protein*
AKT – *Protein kinase B*
ANOVA – *Analysis of variance*
APAF1 – *Apoptotic peptidase activating factor 1*
APC – *Antigen presenting cell*
ARF4L – *ADP ribosylation factor 4 like*

B

BBB – *Blood-brain barrier*
BCNU – *bis-chloroethylnitrosourea*
Bim – *Bcl-2-like protein 11*
Bmi-1 – *B lymphoma Mo-MLV insertion region 1 homolog*
BSA – *Bovine serum albumine*

C

CBF – *Core-binding factor*
CCL2 – *Chemokine (C-C motif) ligand 2*
CCL17 – *Chemokine (C-C motif) ligand 17*
CCL18 – *Chemokine (C-C motif) ligand 18*
CCL22 – *Chemokine (C-C motif) ligand 22*
CCR4-NOT – *C-C chemokine receptor type 4-NOT*
CCND1 – *Cyclin D1*
CCND2 – *Cyclin D2*
CDK – *Cyclin-dependent kinase*
cDNA – *complementary DNA*
CHAPS – *3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate*
CNS – *Central nervous system*
CRAMP – *Cathelicidin-related antimicrobial peptide*
CTL – *Cytotoxic T lymphocites*
CXCL10 – *C-X-C motif chemokine 10*
Cy3 – *Cyanine 3*
C/EBPBβ – *CCAAT-enhancer-binding protein B beta*

D

DAXX – *Death-associated protein 6*
DC – *Dendrytic cell*
DCC – *Deleted in colorectal cancer*
DGCR8 – *DiGeorge syndrome criticical region gene 8*
DIC – *Differential interference contrast*
DIG – *Digoxigenin*
DLS – *Liposomal delivery system*
DMEM – *Dulbecco's modified eagle medium*
DNA – *Deoxyribonucleic acid*
DOGS – *Dioctadecylamidoglycylspermidine*
DOPE – *L-α-dioleoylphosphatidylethanolamine*
dsRNA – *double stranded RNA*
DTT – *1,4-dithio-D-threitol*

E

E – *Embryonic day*
E2F3 – *E2F transcription factor 3*
ECF – *Enhanced chimiofluorescence*
EDTA – *Ethylenediaminetetraacetic acid*
EGF – *Epidermal growth factor*
EGFR – *Epidermal growth factor receptor*
EGFRvIII - *Epidermal growth factor receptor variant III*
eIF4A – *Eukaryotic initiation factor-4A*
ELISA – *Enzyme-Linked Immunosorbent Assay*
EMR1 – *EGF-like module-containing mucin-like hormone receptor-like 1*
eNOS – *Endothelial nitric oxide synthase*
EphA2 – *Ephrin type-A receptor 2*

F

FACS – *Fluorescence-activated cell sorting*
FAM – *6-carboxyfluorescein*
FasL – *Fas (also known as CD95/APO-1) ligand*
FHIT – *Bis-(5'-adenosyl)-triphosphatase*
FISH – *Fluorescence in situ hybridization*
FITC – *Fluorescein isothiocyanate*

G

GALT3 – *βGlcNAc β1, 3-galactosyltransferase, polypeptide 3*
GBM – *Glioblastoma*
GCV – *Ganciclovir*
GDP – *Guanosine-5'-diphosphate*
GFAP – *Glial fibrillary acidic protein*
GFP – *Green fluorescent protein*
GL261 – *Mouse glioma cell line*
GLEA1 – *Glioma expressing antigen 1*
GLEA2 – *Glioma expressing antigen 2*
GMCSF – *Granulocyte macrophage colony stimulation factor*
gp100 – *Glycoprotein 100*
Grb2 – *Growth factor receptor-bound 2*
GTP – *Guanosine-5'-triphosphate*

H

HEK293T – *Human embryonic kidney cell line*
HEPES – *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HER-2 – *Human Epidermal growth factor receptor 2*
HGF – *Hepatocyte growth factor*
HLA – *Human leukocyte antigen*
HNRNPK – *Heterogeneous nuclear ribonucleoprotein K*
HoxD10 – *Homeodomain transcription factor*
HPRT – *Hypoxanthine-guanine phosphoribosyltransferase*
HSV-TK – *Herpes simplex vírus – tyrosine kinase*

I

IFN-γ – *Interferon gamma*
IgG – *Immunoglobulin G*
IgM – *Immunoglobulin M*
IKK α – *Inhibitor-κB kinase alpha*
IKK β – *Inhibitor-κB kinase beta*
IKK ε – *Inhibitor-κB kinase epsilon*
IL-1 – *Interleukin–1*
IL-1R – *Interleukin–1 receptor*
IL-1Ra – *Interleukin–1 receptor antagonist*
IL-1β – *Interleukin–1 beta*
IL-4 – *Interleukin–4*
IL-6 – *Interleukin–6*
IL-10 – *Interleukin–10*
IL-12 – *Interleukin –12*
IL-12p35 – *Interleukin–12 p35 subunit*
IL-13 – *Interleukin–13*
IL-13Rα2 – *Interleukin–13 receptor subunit alpha-2*
IL-23 – *Interleukin–23*
iNOS – *Inducible nitric oxide synthase*
IRAK1 – *Interleukin-1 receptor-associated kinase 1*
IRAK2 – *Interleukin-1 receptor-associated kinase 2*
IRS-1 – *Insulin receptor substrate-1*
IRS-2 – *Insulin receptor substrate-2*

J

JMY – *Junction mediating and regulatory protein*
JNK – *c-Jun N-terminal kinases*

K

KRAS – *V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog*
KLF4 – *Kruppel-like factor 4*

L

LN-5 – *Laminin–5*
LNA – *Locked nucleic acid*

***LOH** – Loss of heterozygosity*
***LPS** – Lipopolyssacharide*

M

***MAGE** – Melanoma-associated antigen*
***MAL –** Myelin and lymphocyte protein*
***MAPK** – Mitogen-activated protein kinase*
***MCS-F** – Macrophage colony stimulation factor*
***MEK** - Mitogen-activated protein kinase kinase*
***MGMT –** O'(6)-methyguanine methyltransferase*
***MHC** – Major histocompatibility complex*
***MICA –** MHC class I polypeptide-related sequence A*
***MICB –** MHC class I polypeptide-related sequence B*
***miRISC** – MiRNA-induced silencing complexes*
***miRNA** – MicroRNA*
***miRNP** – MiRNA-containing ribonucleoprotein complex*
***MMP2** – Matrix metalloproteinase*
***mRNA** – Messenger RNA*
***MT1-MMP** – Matrix metalloproteinase-14*
***mTOR** – Mammalian target of rapamycin*
***MyD88** – Myeloid differentiation primary response gene 88*

N

***N9 –** Mouse microglia cell line*
***N9 21 –** Anti -miR-21 shRNA expressing N9 cells*
***N9 GFP –** GFP expressing N9 cells*
***NED –** N-1-napthylethylenediamide*
***NF-kB** – Nuclear factor of kappa light polypeptide gene enhancer in B-cells*
***NF-kB1/p50** – NF-kB p50 subunit*
***NKG2D** – Natural killer group 2, member D*
***NKp30** – Natural killer cell p30-related protein*
***NKp44** – Natural killer cell p44-related protein*
***NKp46** – Natural killer cell p46-related protein*
***NLR** – NOD-like receptors*
***NO** – Nitric oxide*
***NOD** – Nucleotide-binding oligomerization domain*

O

***OLIG2** – Oligodendrocyte transcription factor 2*

P

***p16 –** Cyclin-dependent kinase inhibitor 2A*
***p21 –** Cyclin-dependent kinase inhibitor 1*
***$p27^{kip1}$ -** Cyclin-dependent kinase inhibitor 1B*
***p53 –** Protein 53*
***p63** – Transformation-related protein 63*
***PAK1 –** Serine/threonine-protein kinase 1*
***PAMP** – Pathogen-associated molecular patterns*
***PBS** – Phosphate buffer saline*
***PCR** – Polymerase chain reaction*
***PDCD4** – Programmed cell death*
***PDGF** – Platelet derived growth factor*
***PDGFR** – Platelet derived growth factor receptor*
***PDK** – Phosphatidylinositol-dependent kinase*
***PE** – Phycoerytrin*
***PGE –** Prostaglandin E*
***PHF-3** – Plant homeo domain-finger protein 3*
***PI3K** – Phosphoinositide-3 kinase*
***PIP_2** – Phosphatidylinositol 4,5-bisphosphate*
***PIP_3** – Phosphatidylinositol 3,4,5-trisphosphate*
***PMSF** – Phenylmethylsulfonyl fluoride*
***PPARγ -** Peroxisome proliferator-activated receptor γ*
***pre-miRNA** – MicroRNA precursor*
***pri-miRNA** – Pre-MiRNA precursor*
***PRR** – Pattern-recognition receptors*
***PTEN** – Phosphatase and tensin homolog*
***PU.1 –** Transcription factor PU.1*
***PVDF** – Polyvinylidene fluoride*

Q

***qPCR –** Quantitative real-time PCR*

R

RB – *Retinoblastoma*
RECK – *Reversion-inducing-cysteine-rich protein with kazal motifs*
RIG – *Retinoic acid-inducible gene*
RISC – *RNA-induced silencing complex*
RLR – *RIG-I-like receptors*
RNA – *Ribonucleic acid*
RNP – *Ribonucleoprotein*
ROI – *Reactive oxygen intermediates*
ROS – *Reactive oxygen species*
RPMI – *Roswell Park Memorial Institute medium*

S

S6K – *S6 kinase*
SART1$_{259}$ ***–*** *Squamous cell carcinoma antigen recognized by T-cells 1*
SART3 – *Squamous cell carcinoma antigen recognized by T-cells 3*
SDS – *Sodium dodecyl sulfate*
SEM – *Standard error of the mean*
SEREX – *Serological expression cloning*
SH2 – *Src homology 2*
SHIP1 – *SH2 domain-containing inositol-5'-phosphatase 1*
shRNA – *Short hairpin RNA*
siRNA – *Small interference RNA*
SMAD3 – *Mothers against decapentaplegic homolog 3*
SMAD4 – *Mothers against decapentaplegic homolog 4*
snord110 – *Small Nucleolar RNA 110*
SOCS1 – *Suppressor of cytokine signaling 1*
SOX2 – *Sex determining region Y-box 2*
SOX6 – *Sex determining region Y-box 6*
SSC – *Saline sodium citrate buffer*
STAT3 – *Signal transducer and activator of transcription 3*

T

TAB2 – *TGF-beta activated kinase 1/MAP3K7 binding protein 2*
TAM – *Tumor associated macrophages*
TAR – *RNA regulatory element*
TBS-T – *Tris buffer saline with tween*
TCR – *T cell receptor*
TFAP2C – *Transcription factor AP-2 gamma*
TGF-β – *Transforming growth factor beta*
TGF-βR – *Transformig growth factor beta receptor*
Tiam1 – *T-cell lymphoma invasion and metastasis 1*
TIMP-3 – *Metalloproteinase inhibitor 3*
TKR – *Tyrosine kinase receptor*
TLR – *Toll like receptor*
TMZ – *Temozolomide*
TNF-α – *Tumor necrosis factor alpha*
TOPORS – *Topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase*
TP53BP – *Tumor protein p53 binding protein*
TRAF6 – *TNF receptor associated factor 6*
TRAIL – *TNF-related apoptosis-inducing ligand*
TRBP – *RNA-binding protein TAR*
tRNA – *Transfer RNA*
TRP2 – *Tyrosinase-related protein 2*
TSA – *Tyramide signal amplification*
TSC – *Tuberous sclerosis complex*

U

ULBP1-3 – *UL16 binding proteins 1-3*

V

VEGF – *Vascular endothelial growth factor*
VEGFR – *Vascular endothelial growth factor receptor*

W

WHO – *World Health Organization*

Abstract

Keywords: Glioma; Microglia; miR-21.

Abstract

Microglia cells are the endogenous immune cells of the central nervous system. These cells play an important role in the defense of brain parenchyma against infectious diseases, trauma, neurodegeneration and brain tumors (such as glioma). It has been reported that glioma cells modulate microglia-mediated immune response in order to promote tumor proliferation. However, the mechanisms underlying such modulation are not yet completely clear. Several miRNAs, such as miR-21 and miR-155, have been reported has being overexpressed after immune activation of microglia and to be involved in the regulation of the immune response. In order to clarify how microglia-mediated immune response may be modulated by glioma cells, N9 microglia cells were incubated with glioma-conditioned medium, derived from GL261 mouse glioma cells, and evaluated the changes in the production of pro-inflammatory mediators. We observed an immune response, characterized by an increase in the mRNA levels of IL-1β and TNF-α (two pro-inflammatory cytokines) along with an increase of the expression of iNOS mRNA and nitric oxide production. Moreover, an increase in the expression miR-21 and miR-155 was observed. In order to unravel the role of miR-21 in the regulation of microglia immune response, N9 microglia cells were transduced with a lentiviral vector encoding a short-hairpin sequence against the mature form of miR-21 (anti-miR-21 lentivirus). This lentiviral vector also provides the cells with the ability to express GFP and, thus using this strategy, we generated N9 cells expressing GFP and low levels of miR-21 (N9 21). N9 cells transduced with a lentiviral vector encoding only GFP were used as control cells (N9 GFP). Following miR-21 silencing, we observed a decrease in the production of the pro-inflammatory cytokine IL-6 and a dramatic decrease in NO production, which suggests that this miRNA is required for a normal microglia-mediated immune response. A non-viral strategy was also employed to silence miR-21 in microglia, which involved the delivery of anti-miR-21 oligonucleotides to N9 microglia cells through DLS lipoplexes. Similar results were obtained with lentiviral- and DLS-mediated miR-21 silencing. Finally, to evaluate whether microglial activation would affect glioma cell proliferation, glioma cells were exposed to conditioned medium produced by N9 cells. Surprisingly, silencing of miR-21 in microglia enhanced glioma cell death through apoptosis when glioma cells were exposed to conditioned medium derived from N9 21 cells. Overall, our findings suggest that miR-21 plays an important role in microglia-mediated immune response, and that the silencing of this miRNA in microglia can contribute to glioma cell death through apoptosis.

Resumo

Palavras-chave: Glioma; Microglia; miR-21.

Resumo

As células da microglia constituem as células imunes endógenas do sistema nervoso central. Estas células têm um papel importante na defesa do parenquima cerebral contra infecções, traumas, neurodegeneração e tumores cerebrais (tal como o glioma). Vários estudos mostraram que as células de glioma conseguem modular a resposta imunitária mediada pela microglia de maneira a promover a proliferação tumoral. No entanto, os mecanismos subjacentes a esta modulação ainda não se encontram totalmente esclarecidos. Vários miRNAs, como o miR-21 e o miR-155, foram reportados estarem sobreexpressos após activação das células da microglia e desempenharem um papel importante na regulação da resposta imunitária. De forma a entender como é que a resposta imunitária mediada pela microglia pode ser modulada pelas células de glioma, células de microglia da linha celular N9 foram incubadas com meio condicionado proveniente de células GL261 de glioma de ratinho, tendo-se avaliado alterações em vários mediadores pró-inflamatórios. Observou-se uma resposta imunitária caracterizada por um aumento dos níveis de RNAm das citoquinas pro-inflamatórias IL-1β e TNF-α, acompanhado de aumentos na expressão do RNAm da iNOS e na produção de óxido nítrico. Foram ainda observados aumentos na expressão de miR-21 e de miR-155. De maneira a tentar perceber em que medida o miR-21 estaria envolvido na regulação da resposta imunitária mediada pela microglia, procedeu-se à transdução das células N9 usando um vector lentiviral codificando um "short-hairpin" contra a sequência madura do miR-21). Este vector conferiu também às células a capacidade expressarem GFP, sendo assim gerada uma linha celular de microglia capaz de expressaando GFP e baixos níevis de miR-21 (N9 21). Como controlo, foi utilizado um vector lentiviral que codificando apenas GFP, gerando-se assim uma linha celular controlo que apenas expressava GFP (N9 GFP). Após o silenciamento do miR-21 na microglia, observou-se uma diminuição na produção da citocina pro-inflamatória IL-6 e de NO nestas células, o que sugere que o miR-21 é importante para uma resposta imunitária normal da microglia. Resultados semelhantes foram observados usando uma estratégia de transfecção não viral para entregar oligonucleótidos anti-niR-21 às células a fim de silenciar o miR-21 (silenciamento por transfecção com lipoplexos DLS). Finalmente, de forma a avaliar em que medida a activação da microglia afecta a proliferação das células de glioma, este tipo de células tumorais foi incubado com meio condicionado das células da microglia. Surpreendentemente, a incubação das células de glioma com meio condicionado proveniente de células da microglia em que o miR-21 foi silenciado, resultou num aumento de morte celular por apoptose. No seu

conjunto, os nossos resultados sugerem que o miR-21 não só é importante para a resposta imunitária mediada pela microglia, como também o seu silenciamento na microglia parece potenciar a morte das células tumorais de glioma.

1 - Introduction

1.1 – Overview of Glioblastoma

1.1.1 – Glioblastoma

Primary brain tumors are neoplasms that develop from the various types of cells that constitute the brain. Approximately 50% of all primary brain tumors originate from the specialized neural cells, called glial cells, and therefore are called gliomas. Astrocytomas, gliomas that arise from astrocytes, are the most common type of primary brain tumors [1]. According to the World Health Organization (WHO), astrocytomas are classified into four grades depending on their growth rate and their ability to infiltrate to the nearby brain tissue [1, 2]. Grade I astrocytomas or Pilocytic Astrocytomas are slow growing and usually spreading astrocytomas. Grade II astrocytomas grow faster than the grade I astrocytomas, but are still considered slow growing tumors. Grade III astrocytomas, also called Anaplastic Astrocytomas, are infiltrating tumors that grow faster than lower grade astrocytomas. Finally, grade IV astrocytomas, or as they are more commonly known, glioblastomas, are the most malignat and fast growing astrocytomas [1, 2]. Glioblastoma (GBM), formerly known as glioblastoma multiforme[3], is the most common and malignant tumor within the central nervous system (CNS) [3-5], constituting between 15 to 20% of all intracranial tumors [5-8].

1.1.2 – GBM classification and incidence rate

GBMs can be classified either as primary (*de novo*) or as secondary (progressive), depending on the origin of the tumor. Primary GBMs arise without prior symptoms or signals of lower grade pathologies (Fig. 1). This type of GBM accounts for the majority of the cases, and is more common in old patients [9]. Secondary GBMs are quite rare and usually appear in patients under the age of 45 years old. They arise from lower grade astrocytomas, being estimated that approximately 70% of all grade II astrocytomas will evolve into higher grade gliomas within 5 to 10 years after diagnosis[9]. GBM represents approximately 2% of all primary tumors with a worldwide incidence rate of 7 over 100.000 individuals per year. According to a recent survey, primary GBMs constitute the majority of GBMs (95%) compared with the secondary forms[10].

Despite intense investigation on this pathology, over the past few decades the majority of patients diagnosed with GBM died within less than one year after diagnosis [10]. Standard treatment consists of surgical removal of the tumor, followed by chemotherapy and radiotherapy. Survival rate varies depending on the age of patients, being nearly 20% for patients below 30 years old, and nearly 5% for patients above 30 years old[11].

1.1.3 – Main molecular characteristics of GBM

GBMs along with the high grade gliomas are diffuse tumors with a high ability to spread to the nearby brain tissue [9]. These tumors are characterized by uncontrolled proliferation, robust angiogenesis, intense resistance to apoptosis and genetic instability such as loss of heterozygosity [9, 12]. Among other genetic mutations, those associated with the cell cycle deregulation, such as the mutation of Retinoblastoma (RB) protein or p53, are of particular importance. RB 1, a gene located in chromosome 13q14 that encodes the RB protein has been shown to be mutated in at least 25% of high grade astrocytomas [13]. The RB protein, when hypophosporilated, binds and blocks the action of the E2F transcription factors family, thus promoting cell cycle arrest [9]. On the other hand, p53 is a well-known tumor suppressor that prevents the propagation of cells with unstable genomes, mainly by arresting the cell cycle in the G1 phase or leading the cell to a program of apoptosis[9, 14].

GBMs are very heterogeneous in cytologic composition, presenting more than one type of cells within the majority of the tumors [3, 15]. Cells of an individual glioma may differ in their morphology, genetics, and biological behavior. This heterogeneity occurs not only within an individual glioma and but also among different gliomas, and it is of particular importance in terms of tumor grading and assessment of therapeutic response and resistance[3]. Necrosis is often present and is associated with higher death rates [15]. There are several immunohistochemical markers important for a correct diagnosis of GBM, the glial fibrillary acidic protein (GFAP) and the oligodendrocyte transcription factor 2 (OLIG2) being the most useful and specific markers for clinical classification of gliomas [9]. GFAP is universally expressed in astrocytic and ependymal tumors but rarely in oligodendroglial tumors. OLIG2 is an oligodendroglial marker, which is CNS specific and universally and abundantly expressed in all diffuse gliomas[9]. Several immunohistochemical stem cell markers, such as CD133, Nestin or Musashi have been demonstrated as being present not only in high grade gliomas, but also in lower grade ones, suggesting the presence of cancer stem cells within the tumor tissue, which corroborates with the recurrence of the disease after conventional treatment[9, 16].

1.1.4 – GBM therapy: Conventional treatment and novel therapeutic approaches

<u>*1.1.4.1 - Conventional treatment*</u>

The standard treatment for GBM begins, when possible, with the surgical resection of the maximal tumor possible followed by the combined action of radiotherapy and chemotherapy [8]. The surgical removal of the complete tumor is impossible in most cases, due

to the fact that glioma tumors grow diffusely and infiltrate frequently into the nearby brain tissue. Nowadays, the 5-aminolevulinic acid (a fluorescent label) is being tested as a tumor contrast agent, in order to help the surgeons to achieve a more accurate image of the tumor and thus remove it more efficiently [8, 17].

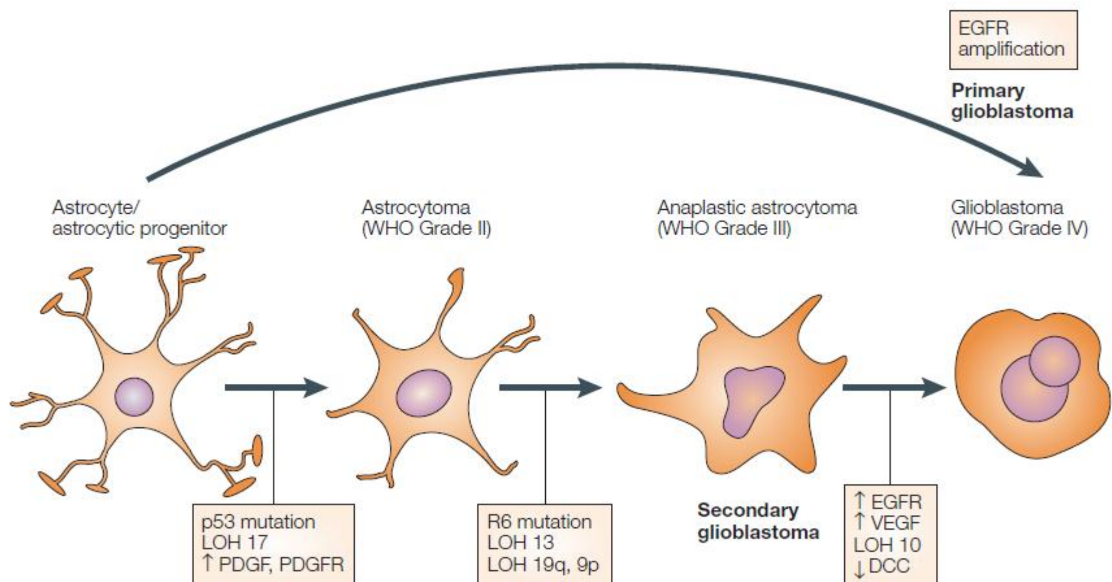

Fig. 1 – GBM. **Primary (or *de novo*) GBMs arise directly from glioma precursor cells**. In contrast, secondary GBMs develop from the progression of low-grade tumors through sequential accumulation of molecular changes [9]. EGFR: Epidermal growth factor receptor; LOH: Loss of heterozygosity; PDGF: Platelet-derived growth factor; PDGFR: Platelet-derived growth factor receptor; VEGF: Vascular endothelial growth factor; DCC: Deleted in colorectal cancer. Adapted from [20]

Currently, the treatment of GBM can be divided into two situations: initial treatment of disease and treatment of recurrent disease. New techniques and new therapeutic agents are always tested first in cases where the disease is recurrent, and their implementation in the initial treatment of the disease is dependent on the results obtained in those first tests [17].

1.1.4.2 – Conventional and new chemotherapeutic agents

The major drugs used for the treatment of GBM are alkylating agents such as temozolamide (TMZ), bis-chloroethylnitrosourea (BCNU) and procarbazine [17-20]. However, many GBMs acquire resistance to these kinds of drugs through demethylation of the promoter of the gene encoding the O'-6-methylguanine-DNA methyltransferase (MGMT), an enzyme

involved in DNA repair [8, 17, 21]. Implantation of biodegradable polymers impregnated with BCNU arose as a new strategy for treatment of GBM after surgery. It has been shown that when combined with fractionated radiotherapy and TMZ, this strategy improved overall survival of the patients several months [8]. Cytostatic drugs are also used in the treatment of GBM such as cis-retinoic acid, thalidomide, tamoxifen or celecobix. These drugs alter the tumor biology preventing its growth and spread, but they do not kill tumor cells by themselves [19].

As GBM biology is unraveled, understanding of their distorted signaling pathways increases and several molecules have been designed to target these pathways. Tyrosine kinase receptors (TKRs), such as the receptors for the epidermal growth factor (EGF), platelet derived growth factor (PDGF) or the vascular endothelial growth factor (VEGF), are important targets in GBM due to their role in tumor growth, spread and angiogenesis [12, 22, 23]. Several drugs that interfere with TKR pathways, either by inhibiting the receptors or other proteins of their pathways, have already been designed (see Fig. 2). Among others, drugs such as sunitinib (an inhibitor of the VEGF receptor) or erlotinib hydrochloride (an inhibitor of the EGF receptor) are already being used in the clinic [12].

1.1.4.3 – Immunotherapy

Immunization against tumors can be very similar to immunization against infections. In a passive immunotherapy approach, the patient is inoculated with *in vitro* activated immune cells, capable of targeting the tumor cells, thus sparing the activation of the patient’s own immune system. On the other hand, an active immunotherapy approach provides a boost to the native immune system of the patient [24].

Over the past few years, the interest in an immunotherapeutic approach for GBM rose significantly, particularly in the field of molecular targets that may turn the tumor more visible to the immune system (also known as immunotherapeutic agents)[25]. GBM genetic instability, although being the main cause of uncontrolled cell growth, may be an open window in what concerns to the appearance of tumor antigens passible of being recognized by immune T cells. Generally, tumor cells contain specific antigens that can be recognized by T cells [25]. However, tumor microenvironments are highly immunosuppressive, due to the presence of anti-inflammatory stimuli, such as interleukin (IL)-4, IL-10, IL-13 transforming growth factor (TGF)-β or corticosteroids [25, 26], thus impairing, not only T cell function, but also the action of other cells (such as macrophages) responsible for innate immune responses [25, 26].

GBMs, like other tumors, are able to overcome host immune defenses through several mechanisms, particularly due to the partial immunologic privilege of the brain, which is a characteristic of note in this kind of tumor [25]. The brain is an immunologically privileged organ due to the presence of the blood brain barrier (BBB). Although activated T cells may cross the BBB and enter the brain in specific situations, the number of cells that remain in an activated state is limited [25]. This is mainly due to the brain environment which is mainly anti-inflammatory and rich in TGB-β, a factor responsible for the downregulation of human leukocyte antigen (HLA) class II in antigen presenting cells (APCs), such as microglia, macrophages or dendritic cells (DCs), which leads to impaired T cell activation through APCs [25, 26]. In addition, studies have shown that infiltrating APCs, although expressing HLA class II, lack the expression of the costimulatory molecules CD80, CD86 and CD40, which are critical for T cell activation[25, 27].

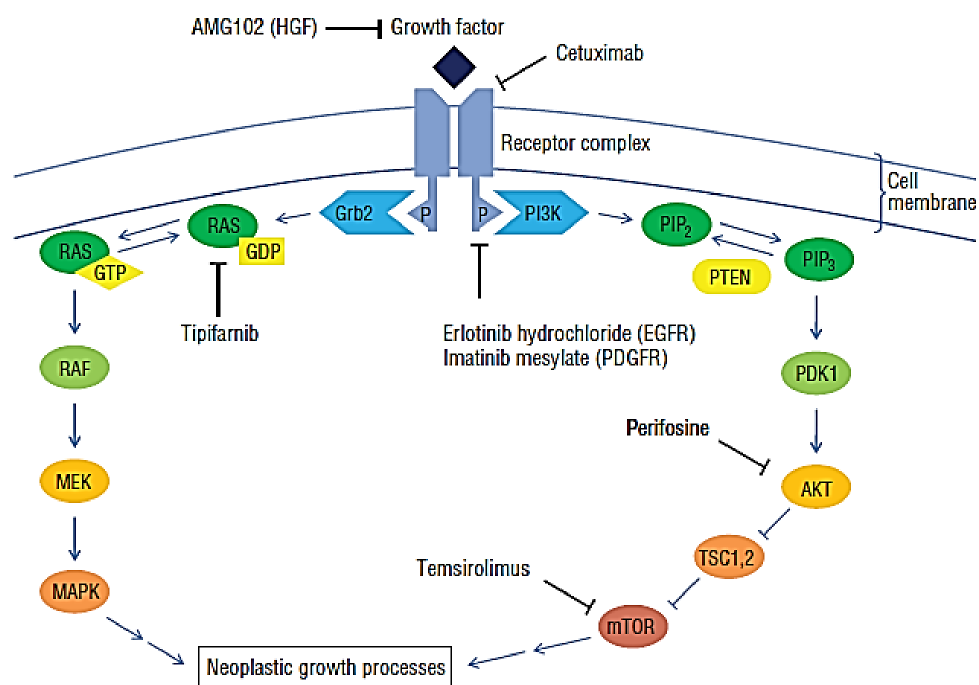

Fig. 2 - Growth factor pathways demonstrating the presence targets for new molecular agents, with examples of agents currently under study. EGFR - epidermal growth factor receptor; GDP - guanosine diphosphate; Grb2 - growth factor receptor-bound 2; GTP - guanosine triphosphate; HGF - hepatocyte growth factor; MAPK - mitogen-activated protein kinase; MEK - MAPK kinase; mTOR - mammalian target of rapamycin; P - phosphate group; PDGFR - platelet-derived growth factor receptor; PDK - phosphatidylinositol-dependent kinase; PI3K - phosphoinositide-3 kinase; PIP2 - phosphatidylinositol 4,5-bisphosphate; PIP3 - phosphatidylinositol 3,4,5-triphosphate; PTEN - phosphate and tensin homologue; and TSC - tuberous sclerosis complex. Adapted from [17].

DCs are the most potent APCs and are commonly used for active specific immunotherapy. With DC based vaccines, tumor regression has been observed in a number of

cancer patients, including GBM patients [28, 29]. The analysis of antigen specific T cells following DC-based therapy also shows a strong increase in cytotoxic T lymphocytes (CTL) in the blood and an overall increase in CTL infiltration in the tumor [25]. Various strategies have been employed in the past in order to load DCs, these including among others, acid-eluted membrane peptides [30], tumor lysates [31] and specific predicted T cell epitopes from known players in glioma biology, such as the mutated form of the EGF receptor, EGFRvIII [32].

1.1.4.4 – Gene Therapy

Gene therapy has its basis in the premise that DNA can be used as pharmaceutical agent, either by modifying an already existing cell gene or through the insertion of new genes into a cell. Gene delivery can be accomplished using a variety of vectors, such as viruses, polymers or liposomes [17]. In gliomas, viral vectors, such as retroviruses and adenoviruses, have been used to deliver suicide and proapoptotic genes, such as the Herpes simplex virus tyrosine kinase (HSV-TK) that converts the prodrug ganciclovir (GCV) into an active drug GCV-monophosphate, which may then be triphosphorylated and inserted into the DNA during replication. This strategy inhibits the action of the DNA polymerase, thus leading to chain termination and cell death [33]. Despite promising, these studies have been discontinued due to low transduction efficiency and other adverse symptoms in the patients [17, 33]. Liposomal vectors, for example, have been used to deliver therapeutic genes in the preclinical setting [17], such as shown in the work of Ramesh and colleagues where they describe cationic liposomes that efficiently deliver therapeutic tumor suppressor genes p53 and Bis (5'-adenosyl)-triphosphatase (FHIT) to lung cancer metastasis in mice [34].

More recently, small RNA molecules, such as small interfering RNAs (siRNAs) or microRNAs (miRNAs), have emerged as promising active agents and targets. Both siRNAs and miRNAs may regulate gene expression at the RNA level by pairing perfectly or imperfectly with their target mRNAs, thus repressing their translation or signaling them to be degraded [35].These small molecules can be complexed with liposomal vectors (siRNAs) or inserted into viral genomic cassettes (miRNA) as a way to promote their delivery into the cell [36]. The combination of miRNA regulation with gene therapy strategies allows to target and potently modulate the expression of endogenous genes, either by downregulation of the gene mRNA or by the silencing of a specific miRNA aiming at downregulating a specific mRNA [36]. In this regard, recent study has shown the silencing of miR-21 (a miRNA known to be overexpressed in GBM [37, 38]) enhances the vulnerability of GBM cells to the TRK inhibitor sunitinib [39].

1.2 – GBM interaction with the immune system

1.2.1 – GBM antigens

T lymphocytes monitor the peptides presented at the cell surface by the major histocompatibility complex molecules, known as human leukocyte antigen (HLA) in humans [40]. As in other types of cancers, it has been proven that GBM cells express several peptides in their membrane and that can be recognized by the T lymphocytes as tumor antigens, thus triggering their activation. Moreover, it was shown that GBM patients exhibit circulating tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) and that CTLs provide selective and effective immune surveillance of the brain, which suggests that GBM is an antigenic tumor [41, 42].

The ideal glioma-associated antigen is one protein or peptide that is expressed only in GBM tissue but not in normal brain tissue. Several studies have already demonstrated the presence of such ideal antigens, such as the work performed by Liu *et al.,* where it is described that primary GBM cells with high-level MAGE-1 or gp100 expression stimulated IFN-γ production by cognate antigen-specific CTL clones [43]. Other important glioma-associated antigens are the EGFRvIII, which results from a mutation of the EGF receptor that originates a glioma-specific mutant protein, or the squamous cell carcinoma antigen recognized by T-cells (SART)-3 protein. Both of these antigens are expressed in GBM, but not in normal brain [44, 45]. A detailed list of human glioma-associated antigens can be found in Table 1.

1.2.2 – GBM microenvironment, tumor infiltrating cells and immune modulation

Glioma tissue contains more than just cancer cells. A considerable amount of nontransformed cells are present within the tumor, the majority of those being immune cells responsible for innate immune responses as microglia (nearly one third of tumor mass), tumor-associated macrophages (TAMs), and mast cells [26, 46-48]. GBM microenvironment is highly rich in growth factors and cytokines that play critical roles, not only in the autocrine stimulation of tumor cells, leading to an increase of protease expression and to an enhancement of cell migration, but also acting as suppressors of the immune system cells, preventing these cells from killing tumor cells [49]. Human GBMs cells secrete factors, such as prostaglandin E (PGE), IL-10, VEGF and TGF-β, which are capable of suppressing several immune functions [25, 49]. TGF-β has been shown to suppress the cytotoxic response of glioma-derived T cells against tumor targets, including autologous glioma cells. Glioma-derived IL-10 has also been shown to downregulate MHC Class II expression on monocytes and suppresses T cell proliferation [49].

Table 1 – Human glioma-associated antigens.

Antigen Type	Gene	Expression in normal tissue	CTL/Antibody reactivity
MHC class I	TRP-2	Yes	Lysis of target cells
	EphA2	Little	Lysis of target cells
	AIM-2	Yes	Lysis of target cells
	$SART1_{259}$	Unknown	IFN-γ release
	SART3	No	Lysis of target cells
	HER-2	Yes	Lysis of target cells; IFN-γ release
	gp100	Little	Lysis of target cells; IFN-γ release
	MAGE-1	No	IFN-γ release
	IL13Rα2	Yes	Lysis of target cells
	ARF4L	Little	Lysis of target cell
	GALT3	Little	Lysis of target cell
	SOX2	Little	Lysis of target cell
	EGFRvIII	No	Lysis of target cells; IFN-γ release
SEREX	SOX6	Little	Autoantibodies detected
	GLEA1	Yes	Autoantibodies detected
	GLEA2	Yes	Autoantibodies detected
	PHF3	Little	Autoantibodies detected
"Innate"	NKG2D, NKp30, NKp44, NKp46 ligands		IFN- γ NK Lysis of CD133+ target cells
	MICA, MICB	Yes	NK cell Lysis of target cells
	ULBP1-3	Yes	NK cell Lysis of target cells

Adapted from [44].

Tumor infiltrating immune system cells have been shown to have a dual role. In a first instance, TAMs and microglia secrete anti-tumor cytokines and interact with T cells to destroy cancer cells. These cells assume a M1 phenotype characterized by high phagocytic ability, production of pro-inflammatory cytokines and antigen presentation, [26, 50]. In addition to secretion of chemotaxic molecules, such the macrophage colony stimulating factor (MCS-F), blood monocytes are attracted to the tumor microenvironment, where they are exposed to anti-inflammatory stimuli released by tumor cells, undergoing differentiation to an alternatively activated type of macrophages, called M2 macrophages. This differentiation process is

promoted by the downregulation of IFN-γ (produced by T cells). These M2 macrophages play a role in the control of the inflammatory response by secreting, along with other molecules, IL-10, which is a cytokine known for its anti-inflammatory properties. However, this phenotype has been shown to be beneficial to tumor development, since it downregulates M1-mediated responses and prevents tumor cell death (see Fig. 3).

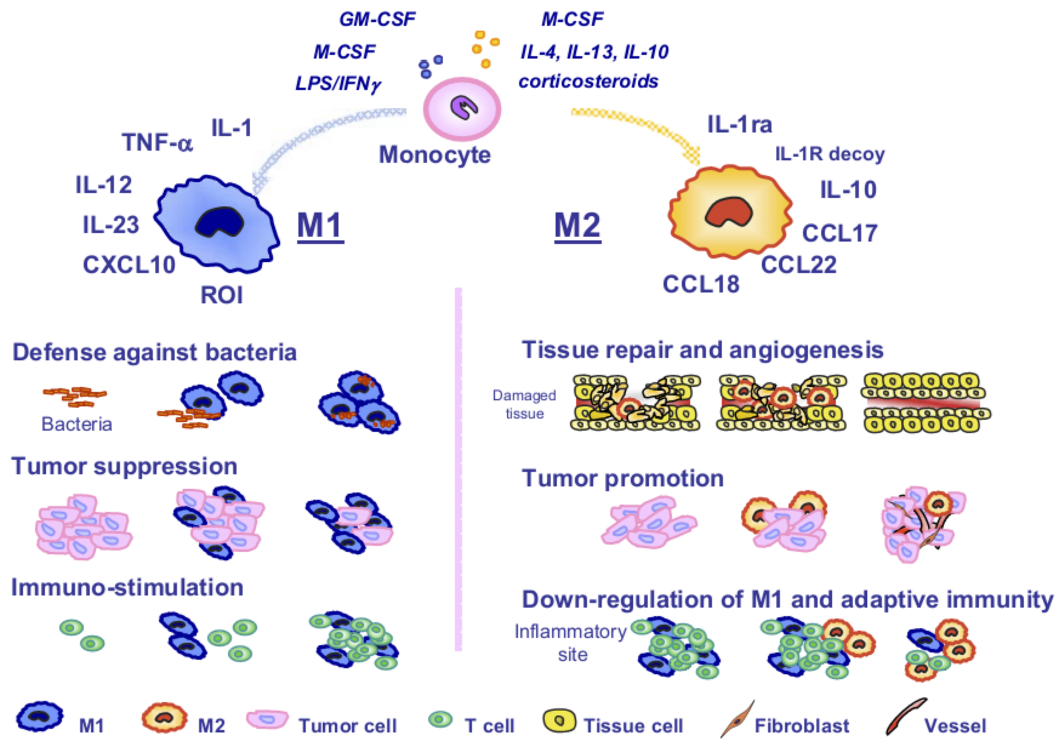

Fig. 3 – Monocyte differential fates. Blood monocyte differentiate into M1 type in the presence of LPS/IFN-γ (classically activated macrophages). M1 macrophages produce high levels of pro-inflammatory cytokines and are characterized by cytotoxic activity against microorganisms and neoplastic cells. On the other hand, when monocytes differentiate in the presence of IL-4, IL-13, IL-10, or corticosteroids, they mature into M2 macrophages (alternatively activated), which secrete amongt others IL-10, which is an anti-inflammatory cytokine. M2 cells are active workers of the host, promoting scavenging of debris, angiogenesis, remodeling, and repair of wounded/damaged tissues. Within the tumor mass, they exert the same functions favoring tumor promotion. In addition, M2 macrophages control the inflammatory response by down-regulating M1-mediated functions and adaptive immunity. Adapted from [26].

1.3 – Overview of microglia

1.3.1 – Microglia

The specialized macrophages of the CNS are called microglia. Microglia is distinguished from neural and other glial cells (such as astrocytes and oligodendrocytes) based on its haematopoietic origin, morphology, gene expression pattern and cellular fuctions [51, 52]. The concept of microglia as a defined cellular element of the CNS was first introduced by Pio del Rio-Hortega in 1932 [52]. Depending on the region of the CNS, microglia constitute 5 to 20% of total glial cells in rodents [53]. Unlike other populations of macrophages, on its resting state, microglia exhibit ramified extensions that emerge from cell body that communicate with the surrounding neurons and glial cells. On the other hand, upon infectious or traumatic stimuli, these cells adopt an amoeboid activated phenotype, and produce many pro-inflammatory mediators, such as cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide, which contribute to the clearance of pathogen infections. However, prolonged or excessive microglial cell activation may result in pathological forms of inflammation that contribute to the progression of neurodegenerative and neoplastic diseases [53, 54]. The role of microglia in health and disease has enhanced the efforts to understand more clearly how their activity is regulated and which mechanisms are involved in such regulation [53].

1.3.2 – Microglia origin and microglial markers

The origin of microglia has been a matter of debate for a long time. It has been proposed that microglia has the same progenitor cells as astroglia in the neuroectoderm [55]. Today, however, it is of general consensus that these cells arise from mesodermal progenitor cells (more specifically myeloid cells) that migrate to the CNS before their complete maturation. Despite this general consensus, microglia origin is still a matter of debate, and the major questions in this field are now focused on the way and type of progenitor cells that migrate to the CNS, and also the time points at which such migration occurs. In a recent review by Kettenmann *et al.* it is suggested that in rodents, microglia originate from monocytic like precursor cells, produced in the bone marrow, which migrate towards the brain until postnatal day 10 [51]. However, in their recent study, Guilhoux and colleagues demonstrated, using fate mapping in mice, that microglia arise from yolk sac progenitor cells around embryonic days (E) 7 and 7.5, enter the embryo around E8 and finally colonize the CNS near E10 (Fig.4) [56, 57]. Moreover, in this study, microglia is identified as an ontogenically distinct population in the mononuclear phagocyte system [56].

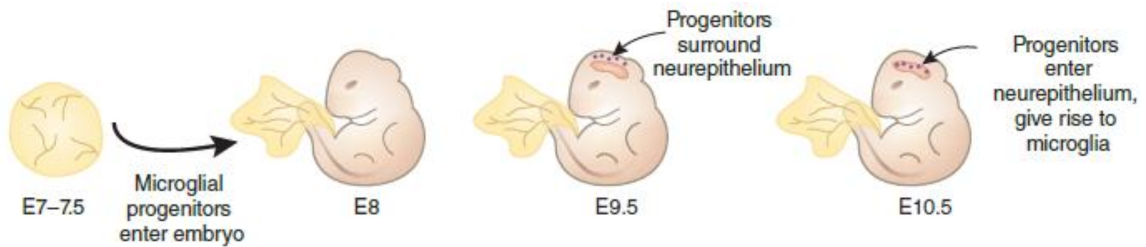

Fig. 4 – **Embryological origins of microglia** as proposed by Guilhoux *et al.*. Microglial progenitors arise in the yolk sac between E7–7.5, enter the embryo at E8 and surround the neuroepithelium by E9.5, between the surface ectoderm and brain rudiment. At E10.5, the earliest microglia are found in the neuroepithelium. Adapted from [57].

Despite the discussion on microglia origin, it is of common consensus that these cells belong to the haematopoietic lineage, sharing the same precursor cells as macrophages or monocytes. Along with DCs, microglia, macrophages and monocytes are classified as mononuclear phagocytes (Fig.5) [58].

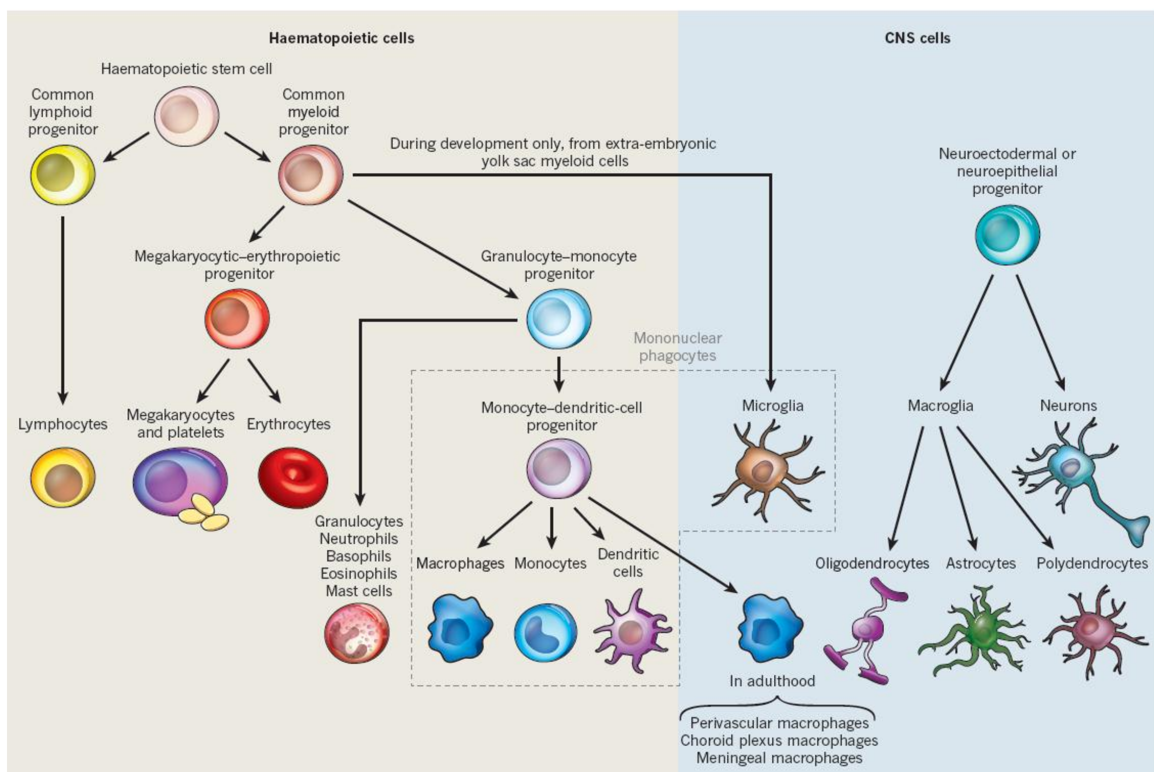

Fig. 5 – **How microglia is related to haematopoietic and CNS cells**. Microglia are the only haematopoietic cells found in the parenchyma of the CNS. Note that the derivation of the perivascular, choroid plexus and meningeal macrophages from the monocyte–dendritic-cell progenitor is speculative and the blood cell from which they originate is unknown. Adapted from [58].

Microglia shares several immunohistochemical markers with macrophages, such as CD11b, CD11c, CD68 and EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1; also known as F4/80 in mice) [53, 59]. However, it is possible to distinguish activated microglia from macrophages based for example on peroxidase activity (present in macrophages but

absent in microglia), and on the expression of CD45 or Laminin-5 (LN-5) wich is higher in macrophages, but low to none in microglia [59]. Table 2 summarizes several consensual markers for distinguishing between microglia and macrophages. It is of general agreement that microglia profile of characterization is: $CD68^+$, CD45 low, $CD11b^+$, CD11c high, MHC class II^+, $CD14^-$ [59].

Table 2 – Selection of several markers used to distinguish microglia from macrophages.

Marker	Microglia	Macrophage
Proliferation	+	-
Peroxidase Activity	-	+
Morphology (as accessed by electronic microscopy)	Spike aspect	Rose aspect
CD11b	+	+
CD11c	+++	+
CD14	+/-	+++
CD68	+++	+++
CD45	+	+++

Adapted from [59]

1.3.3 – Microglia activation and microglia-mediated immune response

1.3.3.1 – Classical microglia activation

Microglial activation is a quick process that can be triggered by a variety of stimuli, such as infection or tissue injury. During the process of classical microglia activation, several changes concerning protein expression and cell morphology occur on microglia phenotype [53, 60]. Microglia expresses pattern-recognition receptors (PRRs), including toll like receptors (TLRs), RIG -I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors, thus acting as the primary sensor for pathogen-associated molecular patterns (PAMPs) in the CNS [53]. Activated microglia upregulates MHC class II expression, required for activation of naive T cells, and produces numerous pro-inflammatory cytokines, including cytokines that induce the differentiation of effector T cells (Fig.6)[53]. Microglia expresses all TLRs, which recognize a variety of different stimuli [61]. As an example, TLR4 along with CD14 recognizes lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria [53, 62]. Microglial activation can be acute or chronic, which has been suggested to depend not only on the duration of an external cue, but also on the specific factor (stress, infection, inflammation, signals from damaged neurons) responsible for the activation process [63].

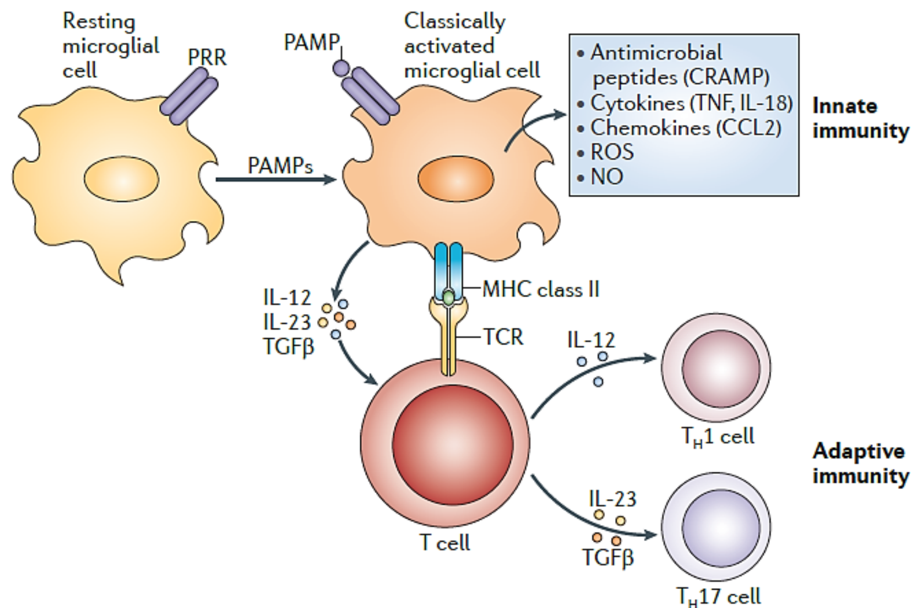

Fig. 6 - Classically activated microglia participates in both innate and adaptive immune responses. Microglia express pattern recognition receptors (PRRs) that recognize various pathogen-associated molecular patterns (PAMPS) found on bacteria and viruses. Following the recognition of PAMPs by microglia, PRR-mediated signalling induces the production of antimicrobial peptides (such as cathelicidin-related antimicrobial peptide (CRAMP)), cytokines (such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β)), chemokines (such as CC-chemokine ligand 2 (CCL2)), reactive oxygen species (ROS) and nitric oxide (NO). These molecules have key roles in innate immunity and are characteristic features of the classical M1-like microglial cell phenotype. Activated microglia also upregulates the expression of MHC class II molecules which allow the presentation of antigens to T cells through the T cell receptor (TCR). In addition, activated microglia produces pro-inflammatory cytokines (such as IL-12) to skew $CD4^+$ T cells into T helper 1 (TH1) cells, or IL-23, IL-6, IL-1β and transforming growth factor-β (TGFβ) to differentiate and activate TH 17 cells. Therefore, classically activated microglia contributes to both innate and adaptive immunity. Adapted from [53].

Morphological changes also occur when microglia is activated. During activation, microglia changes from a ramified to a hyperramified phenotype and subsequently adopts an amoeboid morphology, a process which has been suggested to help microglia invade lesion sites (Fig.7) [60].

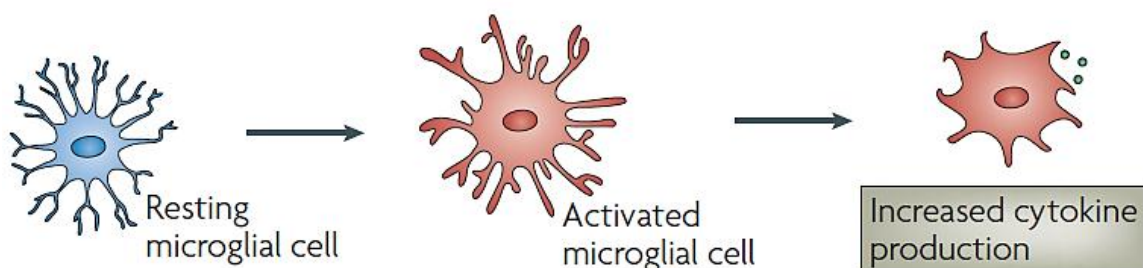

Fig. 7 – Standard profile of microglia morphology during the activation process. Adapted from [54].

1.3.3.2 – Alternative microglia activation

As previously discussed in subsection 1.2.2, it is thought that, like macrophages, microglia also may be activated into two different types of phenotypes, the classical M1 and the alternative M2. Like macrophages, M2 activation of microglia is thought to be induced by IL-4 and IL-10, although there is no clear evidence has been provided [53]. As also previously mentioned, glioma cells are known to produce several immune suppressive factors, such as the anti-inflammatory cytokines IL-4, IL-6 or TGF-β, which are described as driving microglia differentiation into an M2 phenotype (Fig.8). Microglia-like cells are often observed around and within gliomas, however, it is not clear whether these cells are entirely derived from the resident microglial cell population or are macrophages that migrate from the blood stream. Several lines of evidence suggest that glioma-associated microglia might be different from classically activated microglia and more related to alternatively activated macrophages.

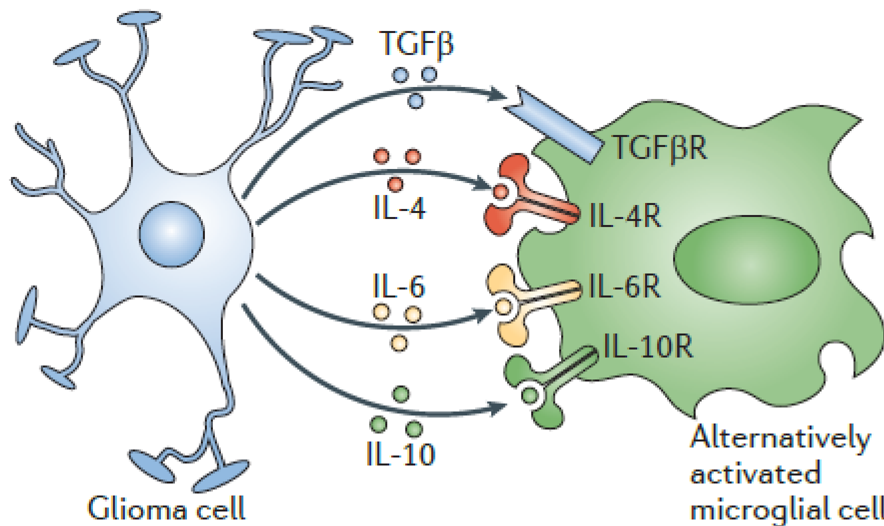

Fig. 8 - Glioma cells secrete factors that induce an M2-like microglial cell phenotype. These factors include transforming growth factor-β (TGFβ), interleukin-4 (IL-4), IL-6 and IL-10.Adapted from [53].

The role of microglia in tumor development has also been a matter of intense debate. In two recent studies, performed by Markovic and colleagues, it was shown that microglia promotes glioma progression by expressing matrix-degrading metalloproteinases (MMPs) such as MMP-2 and MT1-MMP. These MMPs allow cancer cells to easily migrate and invade healthy tissue [64, 65]. In another study, Zhai et al. showed that when microglia first interacts with glioma cancer cells, it becomes activated, although at later time points phagocytosis is significantly decreased and the growth of glioma cells is unaffected. This suggests the presence of immunosuppressive molecules within gliomas [66]. Zhai and colleagues also performed an *in vivo* assay where they ablated the presence of microglia in C57/BL6 mice brains and verified that tumor growth was significantly decreased. This may be explained taking into consideration that

microglia and TAMs are a great source of immunosuppressive cytokines within the tumors and produce MMPs that degrade the cellular matrix, aiding the tumor to invade the surrounding tissues [64-66]. Another two studies performed by Hussain and colleagues showed that microglia isolated from gliomas expresses TLRs and is capable of innate immune responses such as phagocytosis and cytotoxicity, however its capacity to be stimulated via TLRs, secrete cytokines, upregulate costimulatory molecules (CD86, CD80 and CD40), and in turn activate antitumor effector T cells is not sufficient to initiate immune responses [27, 67].

1.4 – MicroRNA biogenesis and function

1.4.1 – Overview of microRNA biology and biogenesis

MicroRNAs (miRNAs) are short noncoding RNAs, 18 to 25 nucleotides long which are expressed endogenously in all eukaryotes. Their history begins in 1993 when Lee, Feinbaum and Ambros discovered that *C. elegans* gene lin-4 did not encode a protein, but two small RNA transcripts instead [68]. These two RNA transcripts were proved to regulate larval development by repressing lin-14 (a gene that encodes a nuclear protein) translation [68]. They proposed that lin-14 regulation was in part due to sequence complementarity between lin-4 and unique repeats within the 3' UTR of the lin-14 mRNA. Seven years later, Reinhart et al. described another miRNA in *C. elegans*, miRNA let-7. This miRNA was similar to lin-4 and also regulated developmental timing in *C. elegans* [69]. At present, we know that these short RNAs are highly conserved gene expression regulators that act by binding mainly to the 3’UTR region of their target mRNAs [70].

1.4.1.1 – Canonical pathway for miRNA biogenesis

MiRNAs are transcribed from the genome by an RNA polymerase II, which originates larger RNA molecules called primary transcripts (pri-miRs). Many of these pri-miRs are polyadenylated and capped after transcription [71] and within these larger RNAs, the mature microRNA sequences form imperfect hair-pin-loop structures. Some pri-miRs transcripts contain clusters of several miRNAs that are processed to their mature forms from a single primary transcript, while others contain only a single microRNA [72]. After being transcribed, pri-miRs are processed by Drosha (an RNase III enzyme present in the nucleus) and by the dsRNA-binding protein DGCR8 (also known as Pasha in invertebrates) originating an RNA molecule approximately 70 nucleotide long called pre-miR (Fig.9). The pre-miRs are then transported to the cytoplasm, by exportin 5, where they are further processed by Dicer, who excises a double stranded RNA with 20-22 nucleotides from the hairpin structure, which is referred as miRNA:miRNA* duplex [70]. This double stranded RNA becomes associated with Argonaute proteins and forms the RISC (RNA induced silencing) complex. Then, the miRNA* (sense strand) is degraded and the complex is directed, by base-pair complementarity, to the target mRNAs of the mature miRNA [70, 71].

MiRNAs interact partially with the 3’ UTR region of the target mRNA, which permits them to have more than one target mRNA, allowing the miRNA to regulate simultaneously ga

large number of genes. This has already been demonstrated experimentally by Grimsom and colleagues [73]. In addition, many genes have predicted target sites for several different microRNAs in their 3' UTRs, as well as multiple target sites for the same miRNA.

1.4.1.2 – Non-canonical pathway for miRNA biogenesis

In 2007, an alternative (non-conventional) pathway, whicht produces miRNA precursors via splicing, was discovered and characterized in invertebrates [74]. The miRNAs originated through this pathway are called mirtrons and are defined by Sibley and colleagues as small RNA molecules with unpaired flanking sequences, immediately adjacent to the splice junctions that are clustered at the outer edge of short introns [75]. Mirtrons are splicing-produced short-hairpin introns that mimic the structural hallmarks of pre-miRNAs and therefore enter the canonical miRNA-processing pathway at a later stage [76].

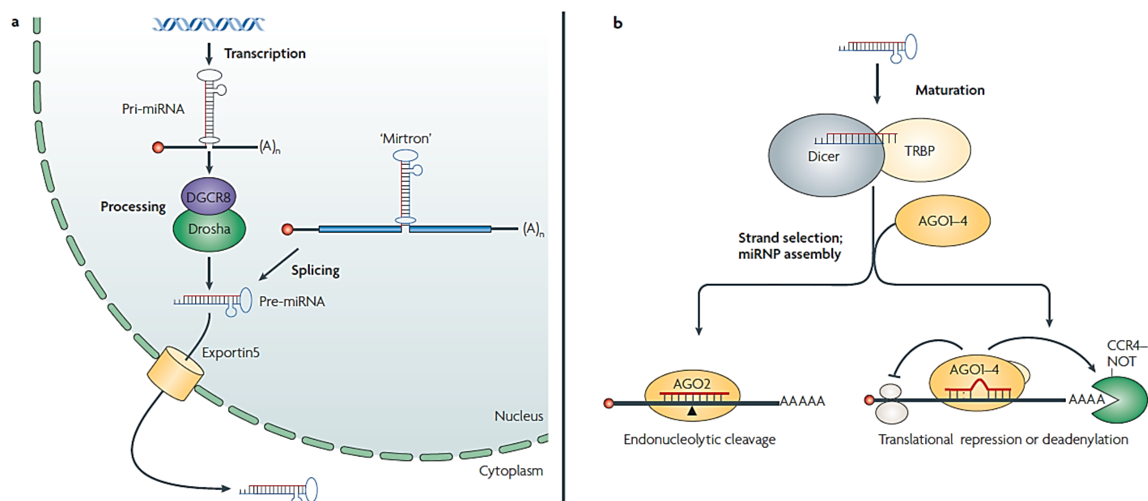

Fig. 9 – Mechanisms of miRNA biogenesis and miRNA-containing ribonucleoprotein complex (miRNP) assembly: **a** - The canonical pathway, involving the production of precursor miRNAs (pre-miRNAs) by Drosha-mediated cleavage of primary miRNA transcripts (pri-miRNA) and the recently discovered non-canonical pathway, involving the production of pre-miRNAs by splicing-mediated cleavage of short-hairpin introns (mirtrons); **b** - Following their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The assembly is a dynamic process, usually coupled with pre-miRNA processing by Dicer, but its details are not well understood. The key components of miRNPs are proteins of the Argonaute (AGO) family. In mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression pathway but only AGO2 functions in RNAi. DGCR8: DiGeorge syndrome criticical region gene 8 protein; TRBP: RNA-binding protein TAR; Ago1-4: argonaute protein 1-4; CCR4-NOT: C-C chemokine receptor type 4-NOT. Adapted from [76].

1.4.1.3 – MiRNA silencing mechanism

In the miRNA 5' terminal exists a sub-region of 6 to 8 nucleotides, called seed region, which is responsible for the specific binding of the miRNA to the mRNA (Fig.10). The seed region binds to the target mRNA at its 3' UTR region. The binding specificity and efficiency of the miRNA:mRNA duplex is modulated by the number and position of the complementary nucleotides. There are always six nucleotides of the miRNA that bind to the 3' UTR of the mRNA, however, if adjacent nucleotides can also bind this will increase the specificity and the efficiency of the recognition [73]. The binding of the miRNA to the mRNA leads to the inhibition of mRNA translation or to the deadenylation and degradation of the mRNA, severely affecting mRNA stability. Therefore, the protein encoded by mRNA undergoes downregulation and its levels in the cytoplasm decrease [70].

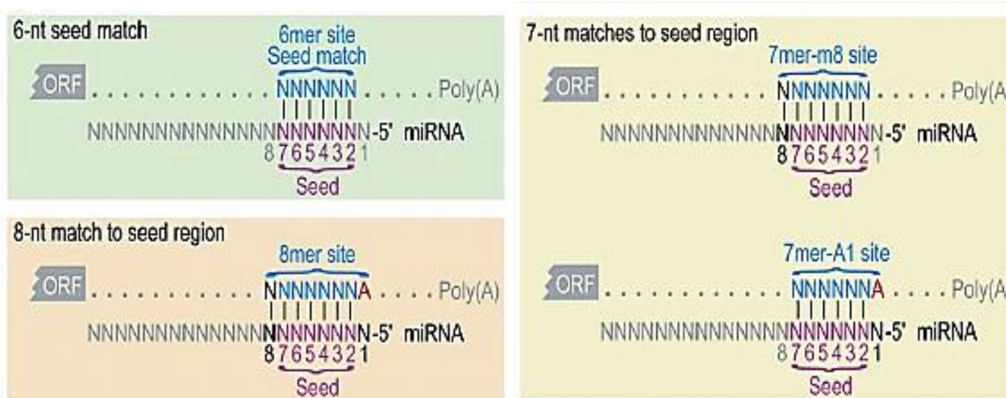

Fig. 10 – Canonical miRNA complementary sites. Adapted from [73].

1.4.2 – The biological roles of miRNAs in glioma

In mammals, miRNAs play a role in the majority of basic cellular processes - such as embryonic development, cell differentiation, metabolism, cell proliferation and cell death - in a wide range of invertebrate and vertebrate organisms (including humans) [77-80]. Widespread influence of miRNAs is also observed in (the regulation of) different physiological responses, including cardiovascular development [81], stem cell differentiation [82] or innate and adaptive immunity [83]. It has also been described that miRNAs have characteristic patterns of expression during brain development and neuronal differentiation [84, 85]. It is thought that at least one-third of all protein-encoding genes in the human genome may be regulated by miRNAs [86]. Therefore, deregulation of physiologic miRNA activity plays an important role in the appearance of several human diseases, including cardiovascular, neurological disorders and cancer [86-88].

1.4.2.1 – MiRNAs involved in gliomagenesis

Some features of carcinogenesis, such as apoptosis, cell proliferation, invasion and migration have been related to miRNA deregulation and several miRNA, such as miR-7, miR-21 or miR-128 have already been described as being deregulated in glioma [37, 89, 90].

MiR-21 has emerged as one of the most consistently highly expressed miRNAs in cancer [72, 89]. In the study of Papagiannakopoulos et al. several tumor supressor protein pathways were identified as targets of this miRNA, such as the TGF-β or the AKT pathways. Direct targets of miR-21 include p53 analogue p63, TGF-β receptors and DAXX (Fig.11), this later being both a mediator of the TGF-β pathway and p53 stabilizer, and involved in the crosstalk of these two apoptotic pathways [37]. Therefore, by targeting of these genes, miR-21 disrupts the normal function of these anti-apoptotic factors, contributing to oncogenesis. Two other mir-21 targets are tissue inhibitor metalloproteinase (TIMP) 3 and reversion-inducing-cysteine-rich protein with kazal motifs **(**RECK). These proteins are inhibitors of matrix metalloproteinases (MMPs) [91].

In their study in 2005, Chan and colleagues showed that miR-21 is highly expressed in GBM. These authors observed that miR-21 knockdown in GBM cell lines triggered caspase activation and associated apoptotic cell death, suggesting an anti-apoptotic function for miR-21. In the same study, it was shown that miR-21 targets programed cell death 4 (PDCD4), which is a tumor suppressor. This protein is thought to be an inhibitor of cell proliferation via p21, which promotes growth arrest [38]. Taken together, these studies suggest that miR-21 is clearly an oncomir that negatively regulates several specific molecules with tumor suppressor functions. This miRNA may play a role in the pathogenesis of several different kinds of cancers, and particularly in GBM. Therefore, miR-21 downregulation can be considered a promising therapeutic approach for human GBM in the future.

MiR-7 is an intronic miRNA that resides in the first intron of heterogenous ribonuclear protein K gene of chromosome 9 and is conserved across all species. This miRNA, whose expression is positively regulated by a homeodomain transcription factor (HoxD10), is downregulated in GBM [92, 93]. Kefas and colleagues showed that miR-7 directly targets EGFR, a receptor known to be upregulated in more than 60% of primary GBMs [89] and that, along with AKT, plays an important role in the development of primary GBM. Its downstream effects of miR-7 downregulation are inhibition of apoptosis and promotion of both cellular proliferation and growth [89]. In their study, Kefas et al., demonstrated that overexpression of miR-7 reduced viability and invasiveness of GBM cells [93].

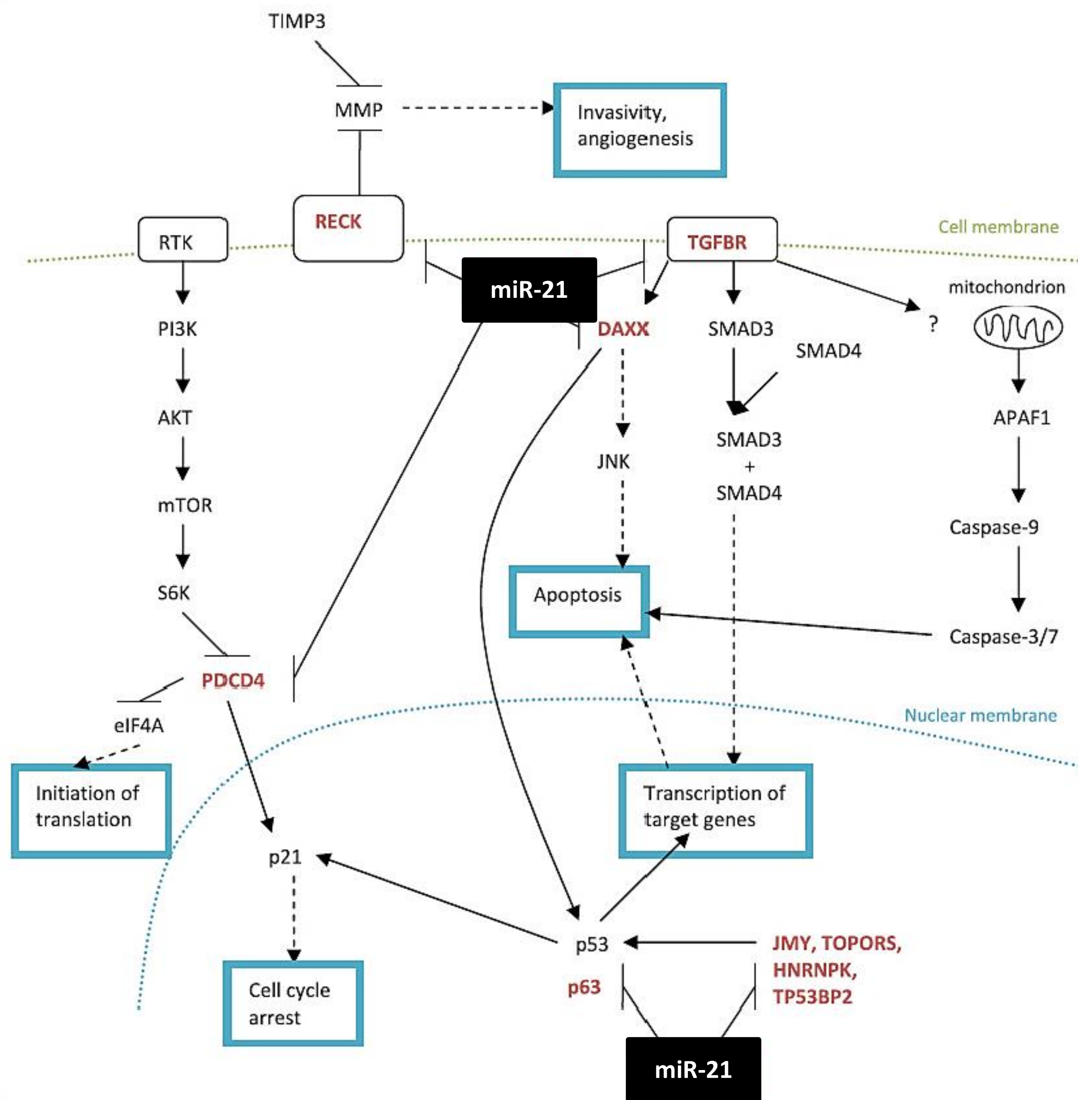

Fig. 11 – Schematic representation of miR-21-influenced signaling pathways. miR-21 inhibits translation of RECK, TGFBR, PDCD4, p63, JMY, TOPORS, DAXX and TP53BP2 proteins, therefore regulating apoptosis cell cycle and translation in glioblastoma. Adapted from [89].

Two other miRNA that are overexpressed in GBM are miR-221 and miR-222. These miRNAs are transcribed as a cluster from chromosome Xp11.3, their expression is coregulated by each other and they also share the same mRNA targets. These two miRNAs promote cell growth and proliferation through the targeting of $p27^{kip1}$[94], which is a protein known to promote cell cycle arrest through the inhibition of cyclin-dependent protein kinases (CDKs) [89].

Several other miRNAs have been described as being deregulated in glioma. Table 3 summarizes several miRNAs that are deregulated in GBM, along with several of their targets.

Table 3 - MiRNAs deregulated in glioblastoma and their validated targets.

miRNA	Type of Expression	Validated Targets	Reference
21	High	PDCD4; TIMP3; RECK; DAXX; p63; TGF-β.	[37, 38, 91]
7	Low	EGFR; IRS-1; IRS-2; PAK1.	[92, 93]
221/222	High	$p27^{kip1}$.	[94]
128	Low	Bmi-1; EGFR; PDGFR.	[90, 95]
195	Low	CCND1; CCND2; E2F3.	[96]
10b	High	HoxD10; Tiam1; KLF4; Bim; TFAP2C; p16; p21.	[97-100]

1.4.3 – The role of miRNAs in the immune response

The immune response is conventionally divided into adaptive and innate. The adaptive immunity, also known as acquired immunity, involves specialized immune cells that mediate a fast response when the body is exposed to a recurrent pathogen. On the other hand, innate immunity provides the first line of defense against various pathogenic infections and is mediated by innate immune cells, such as monocytes, DCs or microglia, and triggers inflammation. Innate immune cells detect PAMPs through several conserved pattern recognition receptors, of which the TLR family is best characterized. For example, TLR3 recognizes double-stranded RNA, which is usually associated with viruses [101], whereas TLR4 recognizes bacterial products, such as LPS [102]. In the recognition of pathogens, TLRs recruit adaptor proteins to facilitate the activation of downstream signalling cascades, such as the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [103, 104]. These signaling processes lead to the expression of inflammatory mediators, such as nitric oxide (NO), cytokines or chemokines, and to the expression of several miRNAs [103, 105]. Recent studies have indicated a role for several miRNAs such as miR-21, miR-146a, miR-125b, miR-155 or miR-9 in the regulation of inflammatory upstream and downstream responses of TLR-activated pathways [106-110]. Table 4 displays a summary of several miRNAs involved in TLR pathways, their targets and main effects on immune cells.

Table 4 – MiRNAs involved in the TLR pathways.

miRNA	Target mRNA	Effect
miR-9	NF-kB1/p50	Transcription by NF-kB is decreased
miR-16	TNF-α	Decreases levels of inflammatory ctyokines
miR-17-5p, miR-20a, miR-106a	Transcription factor CBF	Inhibit of monocyte maturation
miR-21	PDCD4, IL12p35	Derepression of IL-10
miR-27b	PPARγ	
miR-105	TLR2	
miR-106	IL-10	Cooperates with RNA binding proteins to decrease IL-10
miR-125b	TNF-α	
miR-145	MAL	Inhibits TLR signaling
miR-146 a	TRAF6, IRAK1, IRAK2	Negative feedback regulator of TLR signaling
miR-155	AID, MyD88, TAB2, IKKε, SHIP1, SOCS1, C/EBPBβ	Overall proinflammatory, some feedback regulation
miR-199	IKKβ	
miR-221	TNF-α	
miR-223	TLR3, TLR4 IKKα	Granulopoiesis and monocyte activation
Let-7i, let-7e	TLR4	Downregulates inflammatory signaling

Adapted from [111]

<u>*1.4.3.1 – MiRNAs involved in the immune response*</u>

As previously mentioned, a wide range of miRNAs have been reported as being involved in the mechanisms underlying immune response. For these, two functional paradigms have emerged: the pro-inflammatory associated miRNAs, of which miR-155 is the most representative example; and the anti-inflammatory associated miRNAs, such as miR-21 or miR-146a [112, 113]. Despite the array of miRNA reported as being involved in the immune response and in immune cell functions, for the scope of this thesis only miR-155 and miR-21 will be discussed in detail.

1.4.3.1 – a) MiR-155

MiR-155 has been identified as a single primary transcript which is processed from within the second exon of the non–protein-encoding gene, *bic* [114, 115]. This miRNA is involved in B-cell differentiation and proliferation and is probably the best characterized miRNA during B cell response, being upregulated following the activation of these cells in the germinal centre [114]. MiR-155-deficient B cells have defective switching of immunoglobulin (Ig) M to IgG and impaired differentiation into plasma cells, resulting in a dysfunctional humoral response to T cell-dependent antigenic stimulation. It was reported that miR-155 is involved in the downregulation of IgG1 production from B-cells by directly targeting of transcription factor PU.1, which is responsible for the transcription of this immunoglobulin [116, 117]. MiR-155 also targets activation-induced cytidine deaminase (AID), which is also important for antibody production [118]. By targeting PU.1 and AID, miR-155 regulates the differentiation of B cells into plasma cells [117] (Fig.12). Furthermore, knockout mice for miR-155 were shown to be immundeficient and, after immunization, failed to develop a protective response against bacteria [116, 119]. These findings suggest that this miRNA plays an important role in adaptive immunity.

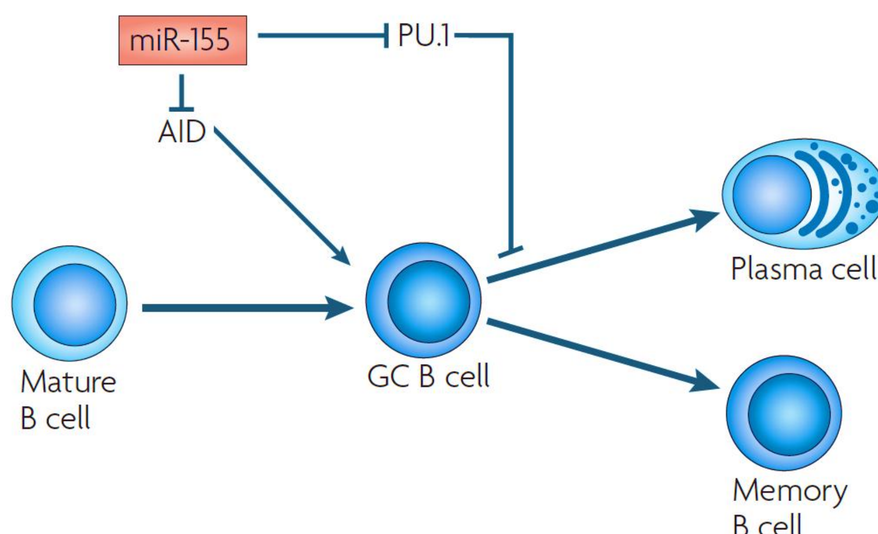

Fig. 12 – MiR-155 regulation of B cell differentiation. MiR-155 targets the transcription factor PU.1 thus leading to the downregulation of IgG1 production and inhibiting the differentiation of B cells into plasma cells. Adapted from [108].

MiR-155 is also involved in the innate immune response since its expression is increased after LPS (via TLR-4) and lipoprotein (via TLR-2) stimulation in monocytes, macrophages or microglia [109, 120]. MiR-155 targets Src homology 2 (SH2) domain-containing inositol-5'-phosphatase (SHIP)1, which is a negative regulator of TLR4 [121] and of the suppressor of cytokine signaling (SOCS)1 [120], thus leading to enhanced AKT activation and IFN production [121, 122].

1.4.3.1 – b) MiR-21

An increase of miR-21 expression has been observed after sustained inflammatory stimuli, similarly to what was found in chronic inflammatory diseases, like lung inflammation due to allergic reactions or ulcerative colitis [123, 124]. It has also been shown by Schetter et al. that the pro-inflammatory cytokine IL-6 induces the expression of miR-21 in a signal transducer and activator of transcription (STAT) 3-dependent manner [125].

MiR-21 has been proposed as playing an anti-inflammatory role in immune cells such as macrophages. In these cells, miR-21 induction via TLR4 was suggested to lead to a decrease of PDCD4, a protein required for NF-kB activation and IL-6 transcription [106]. The down-regulation of PDCD4 has also been shown to be accompanied by an increase in the levels of the anti-inflammatory cytokine IL-10 [106]. McCoy and colleagues demonstrated that IL-10 negatively regulates the TLR4 signaling pathway by targeting miR-155 [126]. As mentioned before, miR-155 targets SHIP1 which is a negative regulator of TLR4. These authors have also demonstrated that IL-10 increases SHIP1 levels by inhibiting miR-155 expression. It has been proposed that the expression levels of miR-155 increase initially due to TLR4 activation, thus resulting in a down-regulation of SHIP1 and TLR4 signaling potentiation. Immediately after TLR4 stimulation, the amounts of PDCD4 within the cells would still be sufficient to activate NF-kB and keep IL-10 levels down. However, as suggested, with the raising levels of miR-21, the levels of PDCD4 would tend to decrease, which may result in a decrease of NF-kB activation and IL-6 expression, and an increase of IL-10 levels. The high levels of IL-10 would then lead to a decrease in miR-155 levels, in a STAT3 dependent manner [126], thereby restoring SHIP1 levels and limiting TLR4 signaling (Fig. 13) [83, 127]. Taken together, these results suggest that miR-21 might have a late anti-inflammatory role in TLR4 mediated immune responses.

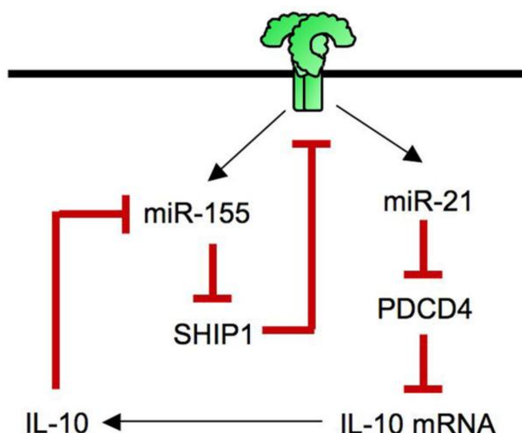

Fig. 13 – Regulation of TLR4 signaling by miR-21 and miR-155. TLR4 signalling increases miR-155 levels, which leads to a downregultion of SHIP1 (a negative regulator of TLR4 signalling). Later, TLR4 activation also increases the level of miR-21, which targets the mRNA PDCD4, thus leading to an increase in the production of IL-10. IL-10 then feeds back on the pathway and specifically inhibits the induction of miR-155, leading to an increase in SHIP1 levels and thus inhibiting TLR4 signalling. Adapted from [127].

2 –Objectives

2.1 – Objectives

- To evaluate the effect of glioma cells on microglia activation, namely microglia mediated immune response to neoplastic cells;

- To evaluate the effect of miR-21 silencing in microglia cells on the immune response mediated by these cells, namely the production of pro-inflammatory mediators and pro-inflammatory miRNA;

- To evaluate the effect of how miR-21 silencing in microglia on glioma cell death.

3 – Methods

3.1 – Methods

3.1.1 – Cell lines and culturing conditions

The GL261 mouse glioma cell line and the HEK293T human embryonic kidney cell line were maintained in DMEM containing 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Paisley, Scotland), 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 10 mM HEPES and cultured at 37°C under a humidified atmosphere containing 5% CO_2. The N9 immortalized mouse microglia cell line was maintained in RPMI-1640 medium (Gibco, Paisley, Scotland) containing 5.4 g/L glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat inactivated FBS (Gibco, Paisley, Scotland), 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma) and cultured at 37°C under a humidified atmosphere containing 5% CO_2.

3.1.2 – Lentiviral production and cell transdution

Lentiviruses encoding the anti-miR-21 shRNA and GFP or a control GFP encoding sequencing were produced in HEK 293T cells with a four-plasmid system, as previously described [128]. The lentiviral particles were produced and resuspended in phosphate-buffered saline solution (PBS) containing 1% bovine serum albumin (BSA). The viral particle content of each batch was determined by assaying HIV-1 p24 antigen using an ELISA kit (RETROtek, Gentaur, Paris, France). The viral stocks were stored at -80ºC until use.

For the lentiviral transduction of N9 cells, cells were plated into 6-well plates at a final concentration of 1×10^5 cells/well. Twenty-four hours after plating, 10 ng of virus coding for either anti-miR-21 shRNA or control GFP were added per $1x10^5$ cells; 8 µg of polybrene (hexadimethrine bromide) were also added to each well in order to increase the efficiency of infection. The cell culture medium was replaced 6 hours after infection and cells were further grown for 48 hours. After this period, cells were plated into 10-cm dishes. Infected cells were selected by growing cells in culture medium containing 1 µg/mL of puromycin. With this procedure, it was possible to generate two new cell lines: one stably expressing GFP and reduced levels of miR-21, which was named N9 21; and another one expressing only GFP (control cell line), which was named N9 GFP.

3.1.3 – Lipoplex preparation and cell transfection

For cell transfection with anti-miR-21 oligonucleotides, lipoplexes were prepared using DLS liposomes, as described previously [129]. Briefly, DLS liposomes were prepared by adding 1 mg of dioctadecylamidoglycylspermidine (DOGS) (Promega, Madison, WI) and 1 mg of dioleoyl phosphatidylethanolamine (DOPE) (Sigma, Munich, Germany) dissolved in 40 µL of 90% ethanol, followed by the addition of 360 µL of sterile H_2O; the mixture was subjected to a short vortex and further incubated for 30 min at room temperature in order to allow liposome formation. The final lipid concentration was 5 mg/ml (2.5 mg of DOGS and 2.5 mg of DOPE). Lipoplexes were prepared by gently mixing 10 µg of oligonucleotides with 76 µL of DLS liposomes containing 190 µg of lipid followed by incubation for 30 min at room temperature. DLS lipoplexes were prepared fresh for every experiment. N9 cells were plated at $1x10^5$ cells/well into 12-well plates, 24h before each experiment. On the day of the transfection, the cell medium was changed to OPTIMEM (Gibco) and the lipoplexes were added to cells, at a final concentration of 100 nM oligonucleotides/well. After a 4-hour incubation period, OPTIMEM was replaced with fresh RPMI medium and cells were further cultured for 24h or 48h.

3.1.4 – Liposomes and lipoplex characterization

DLS liposome and lipoplex size distribution was assessed by photon correlation spectroscopy, using an N5 submicrometer particle size analyzer (Beckman Coulter, Miami, FL, USA). Measurements were made at a 90° angle and at 20°C.

3.1.5 – Assessment of cell transduction/transfection by flow cytometry

To evaluate the extent of cell transduction, N9, N9 21 and N9 GFP cells were plated into 12-well plates at a cell density of 1×10^5 cells/well. Twenty-four hours after plating, cells were washed twice with PBS, detached by exposure to trypsin (5 min, 37°C) and washed once more with PBS. Cells were then ressuspended in 1 mL of cold PBS and immediately analyzed in a FACS Calibur flow cytometer (BD, Biosciences). GFP fluorescence was evaluated in the FL-1 channel and a total of 50.000 events were collected (unless stated otherwise).

To evaluate the efficiency of cell transfection with DLS lipoplexes, N9 cells were plated into 12-well plates at a density of 1×10^5 cells/well 24h before transfection. Twenty-four hours after transfection with FAM-labeled anti-miR-21 oligonucleotides, cells were subjected to the same procedure described above. In order to quench the fluorescence of the lipoplexes attached to the cell surface, which were not internalized, 10 µL of 0.4% Trypan blue were added to 200 µL of cell suspension. FAM fluorescence associated to the anti-miR-21 oligonucleotides was

evaluated in the FL-1 channel and a total of 50.000 events were collected (unless stated otherwise) for both Trypan blue treated and untreated cells.

All data were analyzed using the Cell Quest software (BD).

3.1.6 – Assessment of cell transfection by confocal microscopy

To confirm the extent of cellular internalization of the DLS lipoplexes, N9 cells were plated into ibiTreat 8-well slides (Ibidi, Munich, Germany) at a density of $1x10^4$ cells/well 24h before transfection with DLS lipoplexes. Twenty-four hours after transfection, cells were rinsed twice with PBS, stained with the DNA binding dye Hoechst 33342 (Molecular Probes, Oregon, USA) (1 µg/mL) for 5 min (in the dark), rinsed twice with PBS and maintained alive in this saline solution for image acquisition. Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Germany), using a 60 x oil objective and the Blue (405nm), Green (488nm) and DIC (633nm) lasers. Digital images were acquired using the LSM 510 META software. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

3.1.7 – Evaluation of cell viability following transfection

Cell viability was assessed under the different experimental conditions by a modified Alamar Blue assay. Briefly, 24 h post-transfection, N9 cells were incubated with RPMI containing 10% (v/v) of Resazurin (Sigma, Munich, Germany). After 1 h incubation at 37°C, the absorbance of the medium was measured at 570 and 600 nm. Cell viability was calculated as a percentage with respect to non-transfected control cells, according to equation 1:

$$cell\ viabilty\ (\%\ of\ control) = \frac{[Abs_{570nm} - Abs_{600nm}]}{[Abs^*_{570nm} - Abs^*_{600nm}]} \times 100 \qquad \text{(Equation 1)}$$

Where Abs_{570nm} and Abs_{600nm} are the absorbances of the transfected cells, and Abs^*_{570nm} and Abs^*_{600nm} those of control cells at the indicated wavelengths.

3.1.8 – Conditioned medium production

Two types of microglia conditioned medium were produced: non-activated microglia conditioned medium and activated microglia conditioned medium. In order to produce non-activated medium, fresh RPMI medium was added to N9 cells and collected following 24 h of

contact with the cells. A similar procedure was used to produce activated medium, except that 1 μg/mL of LPS was added to the culture medium. After collection, both conditioned mediums were filtered using a 0.22 μm filter to remove debris, diluted with the same amount of fresh DMEM (glioma culture medium) and kept at 4°C until use.

Glioma conditioned medium was produced by adding fresh DMEM to GL261 cells, and collected after 48 h of contact with the cells. After collection, the medium was filtered using a 0.22 μm filter to remove debris and kept at 4°C until use.

3.1.9 – Nitrite quantification

Nitric oxide production was assessed by the Griess Reagent System (Promega Corporation, Madison, WI), a colorimetric assay that detects the presence of nitrite (NO_2), a stable reaction product of nitric oxide (NO) and molecular oxygen in the cell medium. Briefly, 50 μL of cell medium, collected from each well were incubated for 5 min with 50 μL sulfanilamide, followed by a second incubation of 5 min with 50 μL of N-1-napthylethylenediamide (NED). The optical density of the samples was measured at 535 nm in a microplate reader and the nitrite concentration in each sample was determined by comparison with a standard curve obtained for a solution of sodium nitrite prepared in culture medium. Results were normalized with total protein levels of each sample.

3.1.10 – Extraction of total RNA and cDNA synthesis

Total RNA, including small RNA species, was extracted from N9 microglia cells using the miRCURY Isolation Kit – Cells (Exiqon), according to the recommendations of the manufacturer for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 35 μL RNase-free water by centrifugation. After RNA quantification, cDNA conversion for miRNA quantification was performed using the Universal cDNA Synthesis Kit (Exiqon). For each sample, cDNA for miRNA detection was produced from 20 ng total RNA according to the following protocol: 60 min at 42°C followed by heat-inactivation of the reverse transcriptase for 5 min at 95°C. The resulting cDNA was diluted 40 times with RNase-free water before quantification by qPCR. Synthesis of cDNA for mRNA quantification was performed using the NZY First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) employing 1 μg total RNA for each reaction, by applying the following protocol: 10 min at 25°C, 30 min at 50°C and 5 min at 85°C. After transcription, the samples were further incubated for 20 min at 37°C with an RNase H (from *E. colli*) to specifically degrade the RNA

template in cDNA:RNA hybrids after the first-strand cDNA synthesis. Finally, the obtained cDNA was diluted 10 times with RNase-free water before quantification by qPCR.

3.1.11 – Quantitative real time PCR

Quantitative PCR was performed in a StepOnePlus thermocycler (Applied Biosystems) using 96-well microtitre plates. For miRNA quantification the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon) for miR-21, and miR-155.The small nuclear RNA snord110 was used as a reference. A master mix was prepared for each primer set, according to the recommendations for the real-time PCR setup of individual assays suggested in this kit. For each reaction, 6 µL of master mix were added to 4 µL template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10 µL per well, using the StepOnePlus software (Applied Biosystems). The reaction conditions consisted of polymerase activation/denaturation and well factor determination at 95°C for 10 min, followed by 45 amplification cycles at 95°C for 10 s and 65°C for 1 min (ramp-rate 1,6°C/s).

For mRNA quantification, the iQ SYBR Green Supermix Kit (Bio-Rad) was used. The primers for the target genes (PDCD4, iNOS, IL-6, IL-1β and TNF-α) and for the reference gene (HPRT) were pre-designed by Qiagen (QuantiTect Primer, Qiagen, Hilden, Germany). A master mix was prepared for each primer set, containing a fixed 6.5 µL volume of SYBR Green Supermix and the appropriate amount of each primer to yield a final concentration of 150 nM. For each reaction, 10 µL of master mix were added to 2.5 µL of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 12.5 µL per well, using the StepOnePlus software (Applied Biosystems). The reaction conditions consisted of enzyme activation and well-factor determination at 95°C for 1 min and 30 s, followed by 40 cycles at 95°C for 10 s (denaturation), 30 s at 55°C (annealing), and 30 s at 72°C (elongation).

For both miRNA and mRNA quantification, a melting curve protocol was started immediately after amplification and consisted of 1 min heating at 55°C followed by 80 steps of 10 s, with a 0.5°C increase at each step. The miRNA and mRNA fold change with respect to control samples was determined by the Pfaffl method, taking into consideration the different amplification efficiencies of all genes and miRNAs analysed in each experiment. The amplification efficiency for each target or reference RNA was determined according to the formula: $E = 10^{(-1/S)} - 1$, where S is the slope of the obtained standard curve.

3.1.12 – Fluorescence in situ hybridization (FISH)

FISH was performed in cultured adherent cells as described by Cardoso *et. al* [120]. Briefly, wild type, GFP and 21 N9 cells were plated onto multi-chambered coverglass slides Millicell EZ SLIDE (Merck KGaA, Darmstadt, Germany) appropriate for confocal microscopy imaging. Twenty-four hours after plating, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized at 4°C in 70% ethanol for 4 h. Cells were then incubated with a fresh acetylation solution (0.1 M triethanolamine and 0.5% (v/v) acetic anhydride) for 30 min at room temperature, rinsed twice in TBS and pre-hybridized in the absence of the LNA probe in hybridization buffer (50% formamide, 5 x SSC, 5 x Denhardt's solution, 250 µg/ml yeast tRNA, 500 µg/ml salmon sperm DNA, 2% (w/v) blocking reagent, 0.1% CHAPs, 0.5% Tween) for 2 h at a temperature 22–25°C below the melting temperature (TM) of the probe. The hybridization step was carried out using the DIG-labelled (digoxigenin-labelled) LNA probes for miR-21 at the same temperature overnight. A scrambled probe was used as control. Three stringency washes were performed at the same temperature used for probe hybridization to completely remove the non-hybridized probe. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide in TBS with 0.1% Tween-20 (TBS-T) for 30 min, followed by three washes with TBS-T. The slides were then placed in blocking solution (TBS-T, 10% heat-inactivated goat serum, 0.5% blocking agent) for 1 h at room temperature and incubated for the same period of time with an anti-DIG antibody (Roche, Amadora, Portugal) conjugated with hydrogen peroxidase. To amplify the antibody signal, slides were further incubated with a TSA plus Cy3 (PerkinElmer, Waltham, MA) solution for 10 min in the dark, in accordance with the manufacturer's protocol. The cells were finally stained with the fluorescent DNA-binding dye Hoechst 33342 (Molecular Probes, Oregon, USA) (1 µg/ml) for 5 min in the dark, washed with cold PBS, and mounted in Mowiol (Fluka; Sigma). Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Göttingen, Germany), with a 63 x oil objective. Digital images were acquired using the LSM 510 META software. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

3.1.13 – Western Blot analysis

Total protein extracts were prepared from cultured N9 cells by lysing the cells at 4°C in RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 50 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma), 2 mM DTT and 0.1 mM PMSF. The concentration of protein lysates was determined using the Bio-Rad Dc protein assay (Bio-Rad).

Briefly, 20 µg of total protein were resuspended in loading buffer (20% glycerol, 10% SDS, 0.1% bromophenol blue), incubated for 5 min at 95°C and loaded into a 10% polyacrylamide gel for electrophoretic separation. After electrophoresis, the proteins were blotted into a PVDF membrane, blocked in 5% nonfat milk for 1 hour, incubated overnight at 4°C with an anti-PDCD4 antibody (clone D29C6, Cell signaling; 1:1000) and with the appropriate alkaline phosphatase labeled-secondary antibody (1:20000) (Amersham, Uppsala, Sweden) for 2 hours at room temperature. Equal protein loading was verified by reprobing the membrane with an anti-β-actin antibody (1:10000) (Sigma) and with the appropriate alkaline phosphatase labeled-secondary antibody (1:20000) (Amersham, Uppsala, Sweden) for 2 hours at room temperature. After antibody incubation, the membranes were washed several times with TBS-T (Bio-Rad), incubated with the enzyme substrate ECF (Amersham Biosciences, UK) for 5 min at room temperature and then submitted to fluorescence detection at 570 nm using a VersaDoc Imaging System Model 3000 (Bio-Rad). The analysis of band intensity was performed using the ImageJ software.

3.1.14 – Caspase 3/7 induction assay

Caspase-3/7 activity was assessed using the SensoLyte homogenous AMC caspase-3/7 assay (AnaSpec, San Jose, CA, USA). Briefly, 48 h after incubation with activated or non-activated microglia conditioned medium, derived from all three microglia cell lines, GL261 cells were collected and lysed, according to the instructions of the manufacturer. The cell supernatant and caspase substrate (Ac-DEVD-AMC) were mixed, according to the recommendation of the manufacturer, and further incubated in a black 96-well plate for 40 min at room temperature (under shaking). The production of the AMC fluorophore, released as a result of caspase activity on the substrate, was measured for a period of 5 hours using a microplate reader (SpectraMax Plus 384, Molecular Devices) at excitation/emission wavelengths of 354/442 nm, respectively. Results, presented as relative fluorescence units (RFU), represent the fold change ratio of each experimental condition with respect to control (untreated cells), and were normalized considering the total number of cells in each condition.

3.1.15 – Evaluation of apoptotic cell death

The detection of apoptosis was performed in GL261 cells using the Annexin V-PE Apoptosis Detection Kit (Calbiochem, Merck-Millipore, Darmstadt, Germany). Briefly, 48 hours after incubation with activated or non-activated microglia conditioned medium derived from all three microglia cell lines, cells were detached using trypsin, washed twice with cold PBS and

resuspended in PBS. From this suspension, 1 x 10^5 cells were transferred to 5 mL polystyrene tubes, centrifuged at 1500 rpm for 5 min and resuspended in 200 µL of Incubation Buffer (10 mM HEPES, 150 mM NaCl, and 2 mM $CaCl_2$, pH 7.4). Following this step, the cells in each tube were incubated for 10 min at room temperature with 2 µL of Annexin V-PE and 5 µL of 7-AAD (Sigma). The samples were immediately analyzed in a FACS Calibur flow cytometer. PE fluorescence was evaluated in the FL-2 channel, 7-AAD was evaluated in the FL-3 and a total of 10.000 events were collected per sample. The data were analyzed using the Cell Quest software.

3.1.16 – Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) of at least three independent experiments, each performed in duplicate, unless stated otherwise. One way analysis of variance (ANOVA) combined with the Tukey posthoc test was used for multiple comparisons in cell culture experiments (unless stated otherwise) and considered significant when $p < 0.05$. Statistical differences are presented at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$. Calculations were performed with standard statistical software (Prism 5, GraphPad, San Diego, CA, USA).

4 – Results

4.1 – Glioma cells modulate microglia-mediated immune response

The interaction of glioma with microglia has been a matter of debate in the past few years. Some studies suggest that microglia loses their immune ability when in contact with glioma cells. However other studies suggest that microglia does not lose their innate immune response ability, but their function as antigen presenting cells (APCs) impaired.

In order to clarify how glioma cells modulate microglia mediated-immune response, we incubated N9 microglia cells for 48 h with glioma conditioned medium obtained from GL261 cells and assessed the expression of two pro-inflammatory cytokines, IL-1β and TNF-α (Fig.14a and b) in N9 cells by qPCR. We observed that the levels of the mRNA of both of these cytokines were significantly increased in the presence of glioma cells conditioned medium. Moreover, we observed a slight decrease in the levels of the suppressor of cytokine signaling (SOCS)-1 mRNA (Fig.14c), although this decrease was not statistically significant.

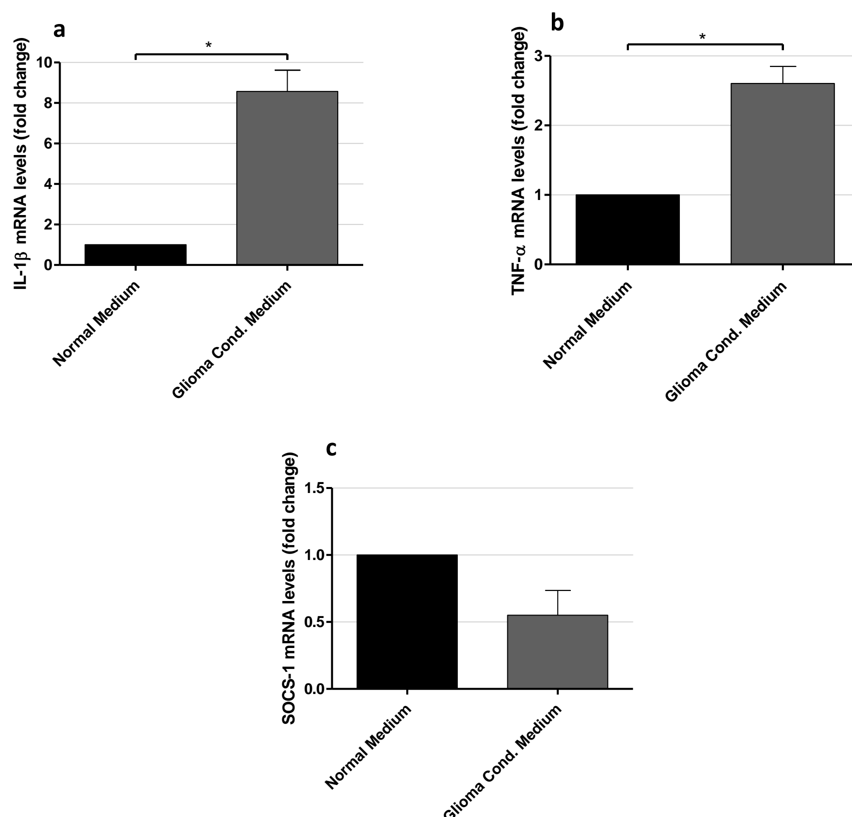

Fig. 14 – Assessment of cytokine production by N9 microglia cells following incubation with conditioned medium from glioma cells. N9 cells were plated 24 h before the 48 h incubation with glioma conditioned medium. **(a)**, **(b)** and **c** – After incubation, total RNA was extracted and the mRNA levels of the pro-inflammatory cytokines IL-1β (a) and TNF-α (b), and the suppressor of cytokine signaling (SOCS)-1 **(c)** were assessed using qPCR. Results are representative of three independent experiments performed in duplicate. Paired one-tailed t-test was used to calculate statistical significance ** – $P < 0.05$.*

In addition to assess cytokine mRNA levels, we quantified, by qPCR the mRNA levels of the inducible nitric oxide synthase (iNOS), an enzyme that is responsible for the production of nitric oxide and known to be upregulated in the presence of certain pro-inflammatory stimulus. A significant increase in the mRNA levels of this protein was observed after N9 cell incubation with glioma conditioned medium, and an even larger increase was detected when the cells were stimulated with LPS following incubation with glioma cells conditioned medium (Fig.15a). Furthermore, and in agreement with the observed upregulation of iNOS, we observed, using the Griess reaction, an increase in the production of nitric oxide in the N9 cells exposed to glioma conditioned medium 24 h before the incubation with LPS (Fig.15b). However, incubation with the conditioned medium by itself was not sufficient to increase NO production in N9 cells, despite the significant increase in iNOS mRNA levels.

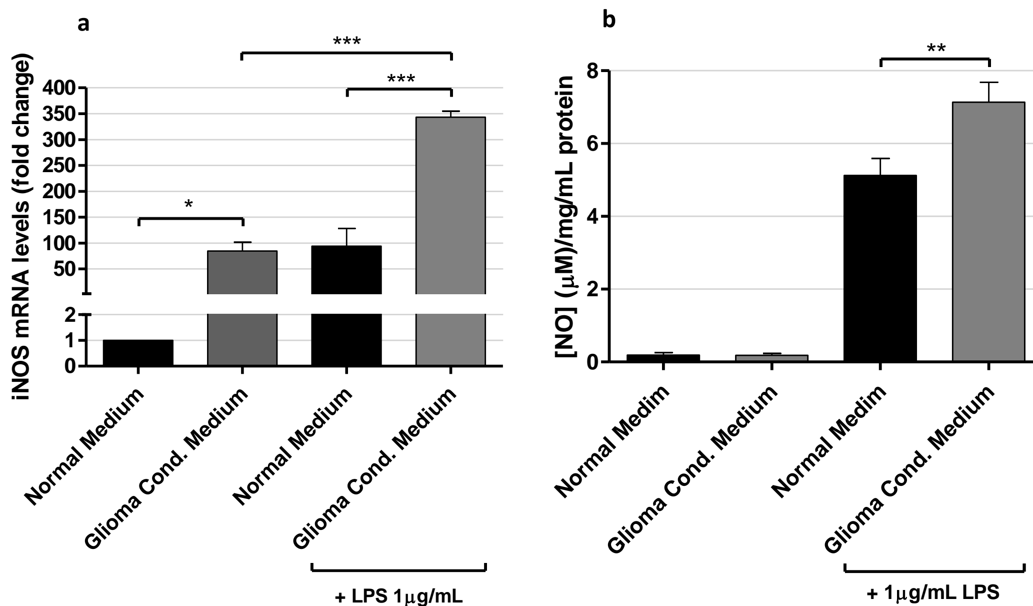

Fig. 15 – Evaluation of iNOS expression and nitric oxide production following N9 incubation with conditioned medium from glioma cells. Twenty-four hours after plating, N9 cells were incubated with glioma conditioned medium. **(a)** – Twenty-four hours after incubation with glioma conditioned medium, 1 μg/mL of LPS was added to the cells, and 24h later, total RNA was extracted and induced nitric oxide synthase (iNOS) mRNA levels were assessed using qPCR. **(b)** – Twenty-four hours after incubation with glioma conditioned medium, 1 μg/mL of LPS was added to the cells. Cells were further incubated with glioma conditioned medium, in the presence or absence of LPS, for more 24h. Twenty-four hours after LPS incubation, NO production was assessed using the Griess test and the NO concentrations obtained were normalized with the total protein levels of each sample. Results are representative of three different experiments performed in duplicate.; ** – P < 0.05, ** – P < 0.01, *** – P < 0.001.*

Finally, using qPCR we assessed the levels of two miRNAs, miR-21 and miR-155. As shown in Fig.16, following incubation of microglia cells with glioma conditioned medium for 48 h, miR-155 was slightly upregulated and miR-21 was significantly upregulated which indicates that both these miRNAs may play a role in the increase of cytokine expression and NO production in the presence of glioma cells conditioned medium.

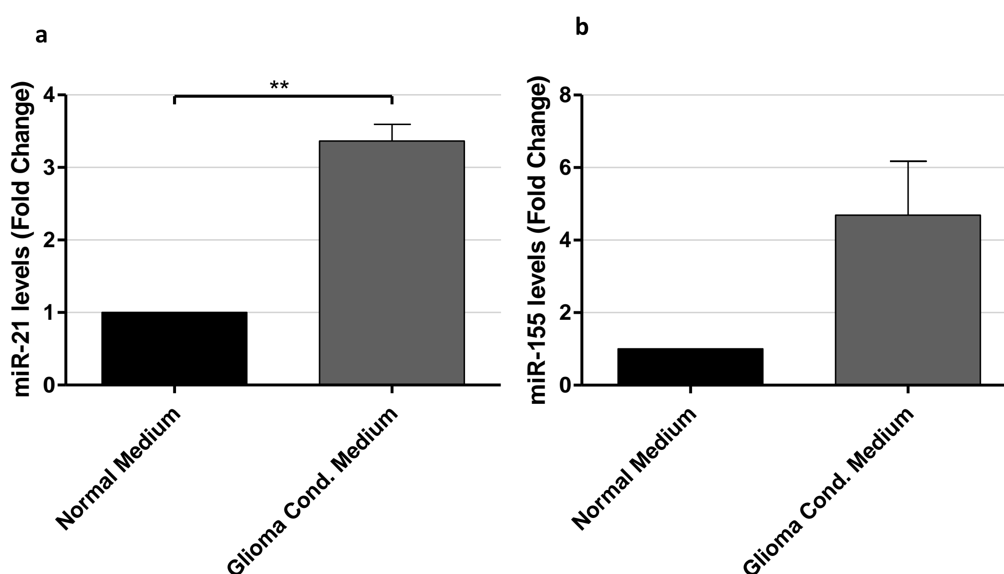

Fig. 16 – Evaluation of miR-21 and miR-155 expression in N9 cells following incubation with conditioned medium from glioma cells. **(a)** and **(b)** – N9 cells were incubated (for 48 h) with glioma conditioned medium. After incubation, total RNA were extracted and miRNAs 21 and 155 levels were determined by qPCR using specific LNA probes for the mature form of these miRNA. Results are presented as miRNA fold change and are representative of three independent experiments; Paired one-tailed t-tes was used to calculate statistical significance; *** – P < 0.01.*

4.2 – MiR-21 silencing influences the microglia-mediated innate immune response

4.2.1 – Evaluation of the lentiviral transduction efficiency of N9 cells

MiR-21 has been suggested to play a role in the regulation of TLR4-mediated immune response. Therefore, in order to clarify if this miRNA also plays a role in the innate immune response against glioma cells, we transduced N9 microglia cells with a lentivirus encoding an anti-miR-21 shRNA and GFP. Control experiments were performed in parallel by N9 cells with a lentivirus encoding only GFP. With this procedure, we generated two new cell lines which we called N9 21 and N9 GFP, respectively. The percentage of transduced cells was assessed by flow cytometry based on GFP expression (Fig.17a and b). N9 21 cells presented approximately 70% of cell transduction, while N9 GFP cells presented approximately 40% of cell transduction (Fig.17b). Transduction of N9 cells with the lentivirus encoding the anti-miR-21 shRNA also conferred them resistance to puromycin, thus allowing to chemically select N9 transduced cells with this antibiotic. On the other hand, the lentivirus encoding only GFP did not confer resistance to this antibiotic and, therefore, the percentage of transduced cells with this lentivirus could not be further increased. However, by testing the transduction of N9 GFP cell line over the course of several weeks, we were able to observe the presence of a stable population of N9 GFP cells (±40%) (Fig.17c).

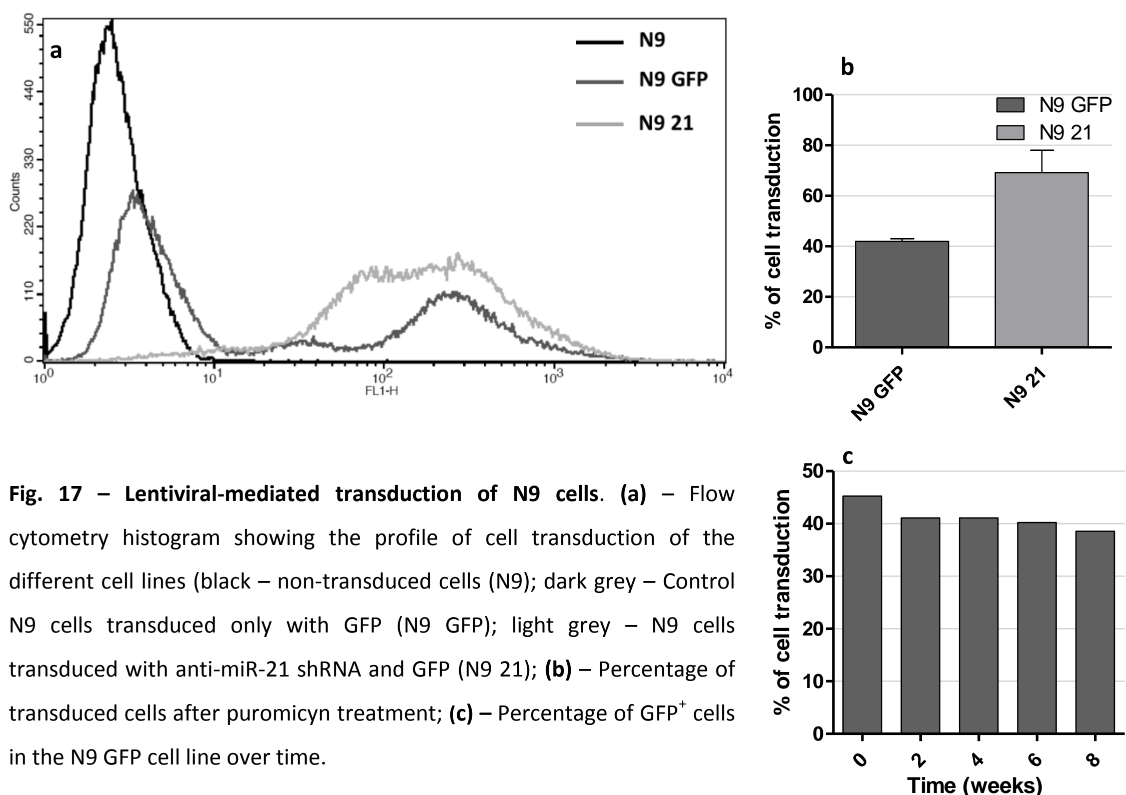

Fig. 17 – Lentiviral-mediated transduction of N9 cells. **(a)** – Flow cytometry histogram showing the profile of cell transduction of the different cell lines (black – non-transduced cells (N9); dark grey – Control N9 cells transduced only with GFP (N9 GFP); light grey – N9 cells transduced with anti-miR-21 shRNA and GFP (N9 21); **(b)** – Percentage of transduced cells after puromicyn treatment; **(c) –** Percentage of GFP^+ cells in the N9 GFP cell line over time.

Quantification of miR-21 expression levels in the wild type and transduced N9 cells by qPCR revealed a significant decrease in the expression of this miRNA in N9 21 cells, with respect to wild type N9 cells, which was not observed in N9 GFP cells (Fig. 18a). These results were in agreement with our observations by FISH using an anti-miR-21 probe (Fig.18b).

As observed, in the presence of an inflammatory-stimulus, such as LPS, miR-21 levels increased in wild type and transduced cells. However, in N9 21 cells, the levels of miR-21 increased in a smaller extent, when compared to control cells (N9 GFP; Fig.18), although this difference was not found to be statistically significant.

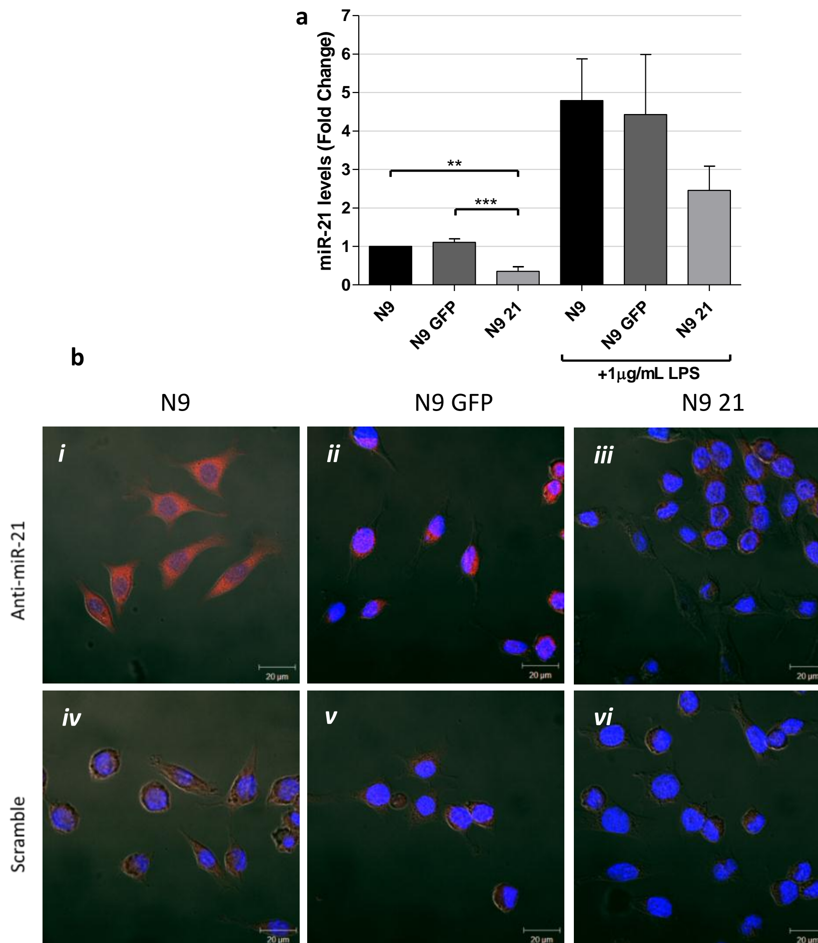

Fig. 18 – Assessment of miR-21 levels in wild type, GFP and 21 N9 cells; Wild type, GFP and 21 N9 cells were incubated for 24 h either in the absence or presence of 1 µg/mL LPS. **(a)** – Following cell incubation with LPS, total RNA was extracted and miR-21 levels were determined by qPCR using specific LNA probes for the mature form of this miRNA. Results are presented as miR-21 fold change with respect to resting N9 wild type cells and are representative of four independent experiments; *$**P < 0.01$ and $***P < 0.001$*. **(b)** – Levels of miR-21 were also analyzed by FISH in non-stimulated wild type (*i*), GFP (*ii*) and 21 (*iii*) N9 cells. MiR-21 was labeled using an anti-miR-21 DIG-conjugated LNA-based probe, specific for the mature form of this miRNA (*i*, *ii* and *iii*). A scramble DIG-conjugated LNA-based probe was used as control (*iv*, *v* and *vi*). Expression of miR-21 (red) was detected using an anti-DIG antibody and the TSA Cy3 signal amplification system. The cell nuclei (blue) were labelled with Hoescht 33342 (Molecular Probes, Oregon, USA). Representative confocal microscopy images of all experimental conditions are presented at a 600 x magnification.

4.2.2 – Evaluation of microglia immune response following lentiviral miR-21 silencing

In order to evaluate the effect of miR-21 silencing in microglia-mediated immune response, N9 cells were challenged with inflammatory stimulus, and the quantification of nitric oxide (NO) production and cytokine expression were assessed. We observed that, 24h after stimulation with LPS, NO production of N9 21 cells was significantly reduced with respect to wild type (N9) and control cells (N9 GFP; Fig. 19a). Regarding the expression of pro-inflammatory cytokines, a significant decrease in the levels of the pro-inflammatory cytokine IL-6 was observed in resting N9 21 cells, with respect to resting N9 GFP cells. Despite the differences revealed in the resting state no significant differences were observed when the different cell lines were activated through incubation with LPS. However, in stimulated N9 21 cells, IL-6 mRNA levels were still lower than those observed in N9 GFP (Fig.19b). In addition, we were not able to observe significant differences in the mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IFN-β (data not shown for TNF-α and IFN-β) between N9 GFP and N9 21 cells, either in resting or LPS-stimulated conditions (Fig.19c).

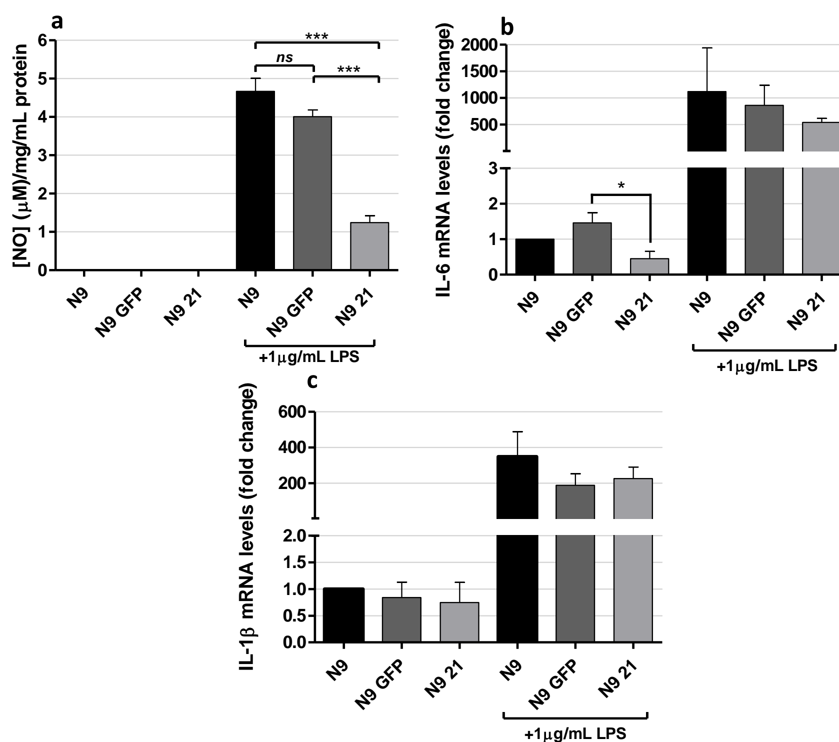

Fig. 19 – Effect of miR-21 silencing in the pro-inflammatory response of wild type and lentiviral transduced N9 cells. Wild type, GFP and 21 N9 cells were incubated for 24h, either in the absence or in the presence of 1μg/mL LPS. Following cell incubation with LPS **(a)** nitric oxide levels were assessed using the Griess test and the NO concentrations obtained were normalized with the total protein levels of each sample; **(b)** IL-6 and **(c)** IL-1β mRNA levels were determined for all cell lines in both resting and activated conditions by q-PCR. Q-PCR results are presented as mRNA fold change with respect to resting N9 wild type cells; Results are representative of four independent experiments. *ns – non-significant, * – $P < 0.05$, *** – $P < 0.001$.*

Taken together, these results show that miR-21 is involved in the nitric oxide production pathway and is important for IL-6 expression in N9 cells. However, miR-21 silencing does not affect IL-1β (and other cytokines) levels, both in resting and LPS-activated microglia, which suggests that this cytokine is not directly regulated by miR-21.

4.2.3 – Evaluation of PDCD4 levels following miR-21 silencing

PDCD4 is a validated direct target of miR-21 and has been also been suggested to be involved in the regulation of IL-10 production after TLR4 stimulation. Therefore, we evaluated the mRNA and protein levels of PDCD4 in N9 cells following miR-21 silencing. As illustrated in Fig.20, an increase in both PDCD4 mRNA and protein levels was observed in resting N9 21 cells, with respect to control cells (N9 GFP). In addition, 24 h after LPS stimulation, a significant decrease in both PDCD4 mRNA and protein levels was obtained, in the three cell lines, which is in agreement with the results previously reported by Sheedy *et. al.* [106]. Despite this decrease, which was expected due to the increase of miR-21 levels in the three cell lines in the presence of LPS (Fig.18a), PDCD4 mRNA levels were significantly higher in activated N9 21 cells, with respect to activated N9 GFP cells (Fig.20a), which may be attributed to the difference in miR-21 levels in these two cells lines. However, following miR-21 silencing, no significant differences in PDCD4 levels between LPS stimulated N9 21 and N9 GFP cells could be detected (Fig.20b and c

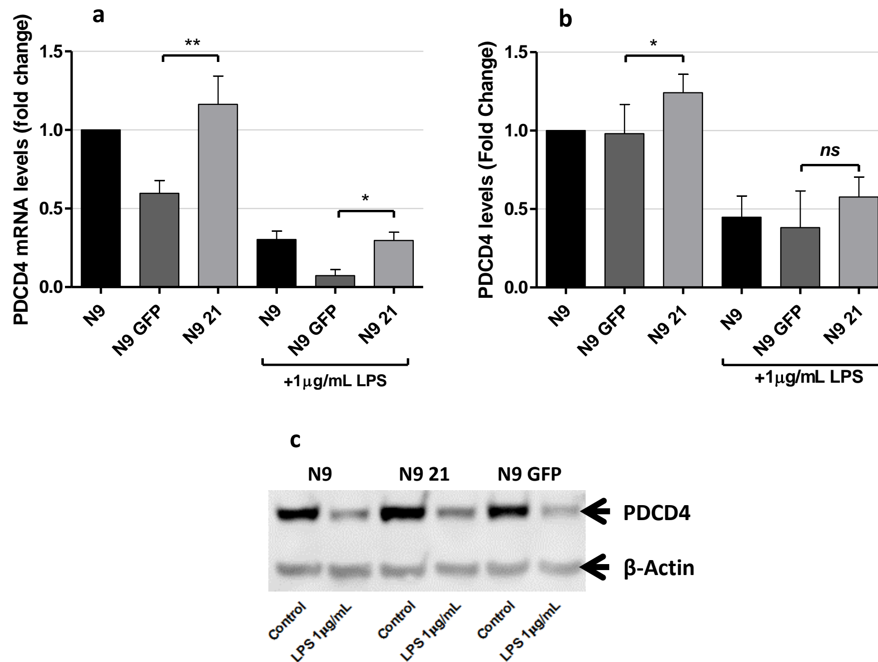

Fig. 20 – Assessment of PDCD4 levels following miR-21 silencing. Wild type, GFP and 21 N9 cells were incubated in the absence or presence of 1µg/mL LPS for 24h. After cell incubation, total RNA and protein were extracted. **(a)** PDCD4 mRNA levels were quantified using q-PCR and **(b)** PDCD4 protein levels were determined using western blot. Results are presented as mRNA or protein fold change with respect to resting N9 wild type cells; **(c)** Representative western blot image showing PDCD4 levels in all cell lines in resting and activated conditions. Results are representative of three independent experiments. *ns – non-significant, * - P < 0.05, ** - P < 0.01.*

4.2.4 – Transfection of N9 cells with DLS lipoplexes

Although lentiviruses have the ability to efficiently transduce the N9 cell line, the expression of the anti-miR-21 shRNA in these cells might not be sufficient to clearly assess the role of this miRNA in the microglia-mediated immune response. Therefore, in order to obtain a stronger, although transient silencing of miR-21, N9 cells were transfected with DLS lipoplexes at a final anti-miR-21 oligonucleotide concentration of 100 nM. DLS lipoplexes have already been described by us and others as being an efficient vector for the delivery of antisense oligonucleotides to different cell lines [129].

Before performing the transfection experiments, DLS liposomes and lipoplexes were characterized in terms of their size, since this parameter plays an important role in transfection efficiency. The size of liposomes and lipoplexes was assessed using photon correlation spectroscopy. Freshly prepared DLS liposomes and DLS lipoplexes obtained from freshly prepared DLS liposomes exhibited a unique particle population with a size of approximately 200nm and 270 nm, respectively. On the other hand, one week old DLS liposomes exhibited two distinct populations of particles; one major population (81.2%) of approximately 270 nm, and a smaller one (18.8%) of approximately 50 nm. DLS lipoplexes prepared with one week old DLS liposomes exhibited a unique population of particles with approximately 240 nm in size (Fig. 21).

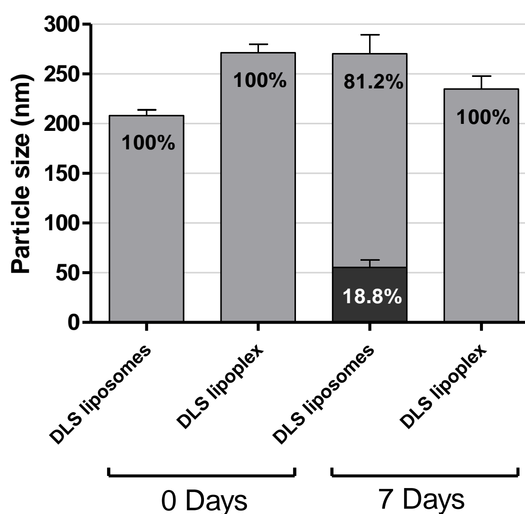

Fig. 21 – **Physicochemical characterization of DLS liposomes and lipoplexes**. Analysis of DLS liposome and lipoplex size was performed immediately (Day 0) and 7 days (Day 7) after liposome preparation. DLS lipoplex were produced from Day 0 or Day 7 DLS liposomes and size analysis was performed immediately after preparation. Particle size was assessed through photon correlation spectroscopy using a N5 submicrometer particle size analyzer (Beckman Coulter, Miami, FL, USA).

As shown in Fig.22, following 24 h incubation of DLS lipoplexes (complexes of liposomes with FAM-labeled anti-miR-21 oligonucleotides) with N9 cells, approximately 85% of lipoplex internalization was achieved as assessed by flow cytometry (Fig.22a and c). Similar results were obtained from parallel experiments in which trypan blue was added to quench the fluorescence of the non-internalized lipoplexes, which suggest that the majority of the lipoplexes bound to the cell surface were internalized (Fig.22b and c). Efficient lipoplex internalization and efficient endosomal release of the anti-miR-21 oligonucleotides 24 h after transfection were confirmed through confocal microscopy analysis (Fig.23).

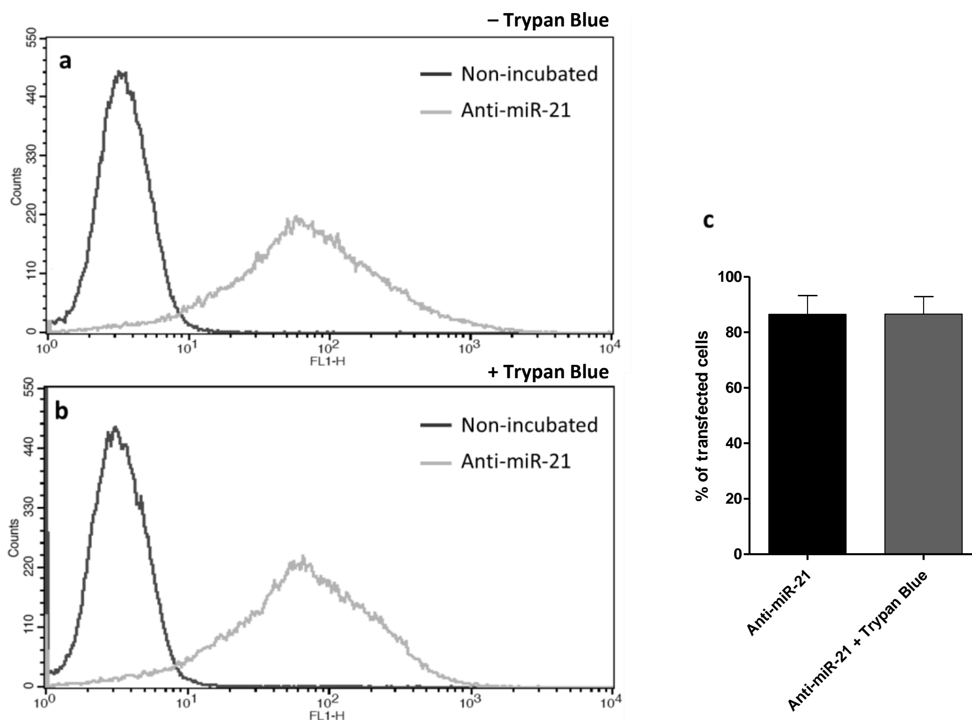

Fig. 22 – Evaluation of DLS lipoplex internalization in N9 cells. N9 cells were transfected with DLS liposomes complexed with FAM-labeled anti-miR-21 oligonucleotides, at an oligonucleotide final concentration of 100 nM. **(a)** The percentage of cells that internalized the DLS lipoplexes was assessed by flow cytometry 24h after transfection.. **(b)** Following the first acquisition of 50.000 events per sample and in order to quench the fluorescence of non-internalized lipoplexes, 10 µL of trypan blue were added to 200 µL of cell suspension and a second acquisition was performed for the same number of events. **(c)** Flow cytometry results are presented as the percentage of cells that bind and internalized FAM-labelled oligonucleotides.

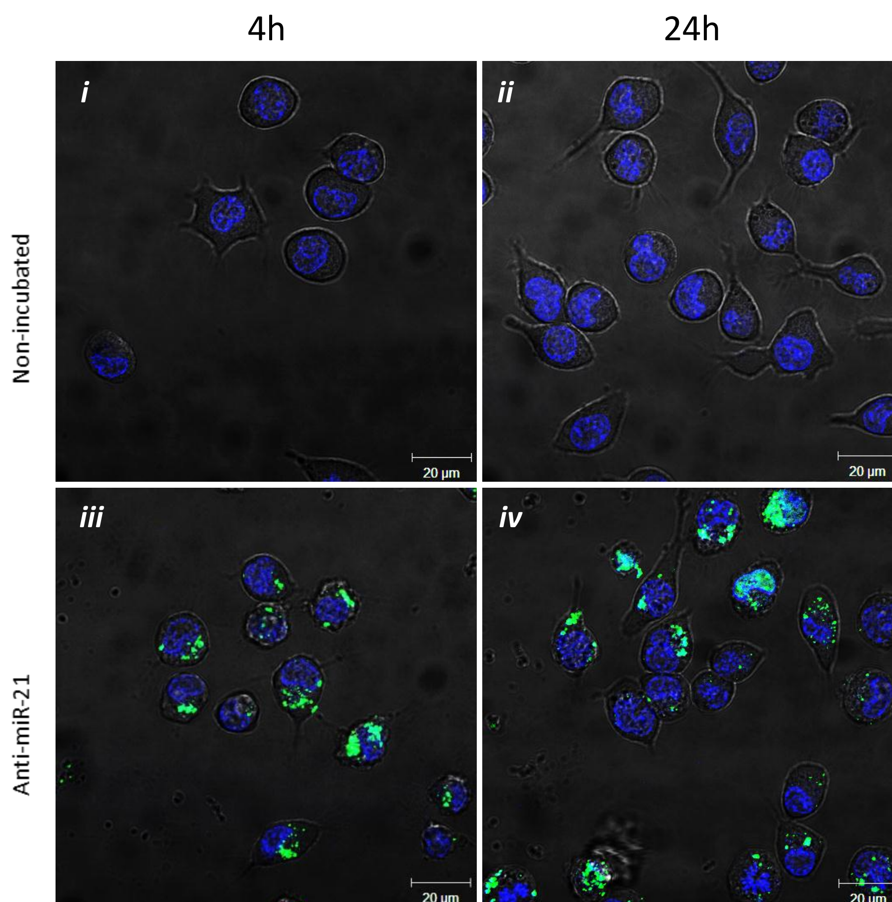

Fig. 23 – Evaluation of DLS lipoplex internalization and cytoplasmic oligonucleotide release in N9 cells. Confocal microscopy was used to confirm lipoplex (green) internalization and efficient endosomal realese 4h (*i* and *iii*) and 24h (*ii* and *iv*) after transfection, Cell nucleus (blue) were stained with Hoechst 33342 (Molecular Probes, Oregon, USA). Representative confocal microscopy images of all experimental conditions are presented at a 600 x magnification. Results are representative of three independent experiments.

Quantification of miR-21 levels by qPCR revealed that the expression of this miRNA was completely abolished in N9 cells transfected with DLS liposomes complexed with FAM-labeled anti-miR-21 oligonucleotide (at a final concentration of oligonucleotide of 100 nM), with respect to the non-transfected or the scramble-transfected cells (Fig.24). As shown in Fig.24, after cell incubation with LPS, an increase in miR-21 levels could be observed in non-transfected cells, but not in cells transfected with anti-miR-21 oligonucleotides, suggesting that miR-21 was completely silenced even in the presence of a strong activating stimulus. However, after LPS stimulation, a decrease in miR-21 levels was observed in cells transfected with scramble oligonucleotides with respect to control cells. This suggests the presence of unspecific effects due to the DLS transfection process or to the fact that the scramble oligonucleotide employed in these experiments was able to interact with miR-21 (Fig.24).

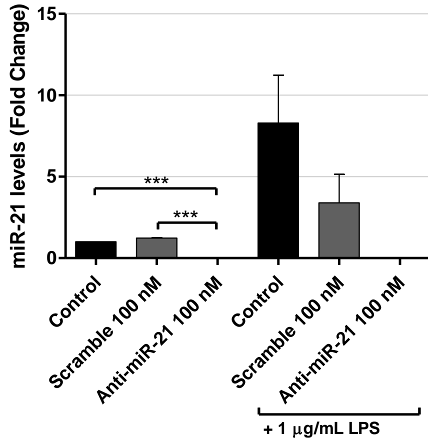

Fig. 24 – Quantification of miR-21 in cells transfected with DLS lipoplex. N9 cells were transfected, 24 h after plating, with DLS liposomes complexed with FAM-labeled anti-miR-21 oligonucleotides or scramble oligonucleotides, at an oligonucleotide concentration of 100 nM. Twenty-four hours after transfection, the cells were stimulated with 1 µg/mL LPS. Total RNA was then extracted and miR-21 levels were assessed in transfected and non-transfected cells by qPCR. Results are representative of three independent experiments. **** - P < 0.001.*

4.2.5 – Evaluation of cell viability following transfection with DLS lipoplexes

Although DLS lipoplexes have been successfully applied for intracellular delivery of single strained oligonucleotides, cationic formulations can be toxic to cells, depending on the cell type. To evaluate whether DLS lipoplexes were toxic to N9 cells, cell viability was assessed following transfection by the Alamar Blue assay. As illustrated in Fig.25, when N9 cells incubated with DLS lipoplexes at a final concentration of 100 nM (the highest oligonucleotide concentration employed), a decrease in cell viability of nearly 30% was observed, both in anti-miR-21 and scramble transfected cells. The cytotoxicity mediated by DLS lipoplexes was found to be dose-dependent, a decrease being observed with decreasing of the final oligonucleotide concentration (Fig. 25). These results may help to explain the unspecific decrease in miR-21 expression levels observed following transfection with the scramble oligonucleotide (Fig.24).

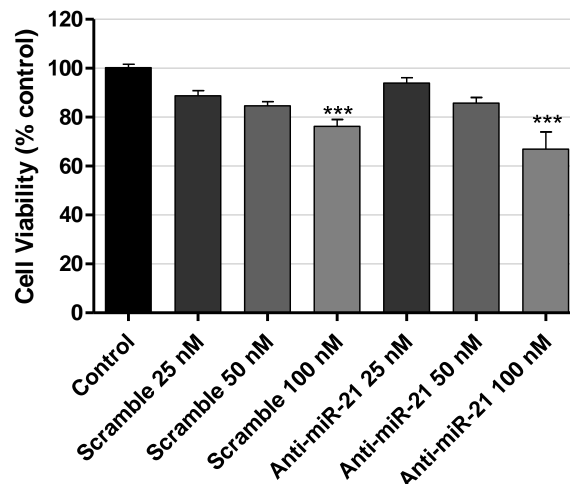

Fig. 25 – Assessment of cell viability. Twenty-four hours after plating, N9 cells were transfected with DLS lipoplex at an oligonucleotide final concentration of 25, 50 or 100 nM. Twenty-four hours after transfection, N9 cell viability was assessed using the Alamar Blue viability assay. Results are representative of two independent experiments performed in triplicate. **** – P < 0.001*.

4.2.6 – Microglia-mediated immune response upon miR-21 silencing via DLS lipoplex

Following the observation of a complete miR-21 silencing mediated by DLS lipoplexes in N9 cells, we further assessed how this miRNA could be important for microglia-mediated immune response. As shown in Fig.26 miR-21 silencing resulted in a reduction of NO production in LPS-incubated N9 transfected cells, which was dependent on the anti-miR-21 oligonucleotide final concentration (Fig.26a). However, we were not able to observe significant differences between anti-miR-21 and scramble conditions, probably due to unspecific effects of the scramble sequence (Fig.24). Taking into account that no significant decrease was detected in the viability of cells transfected with scramble oligonucleotide at final concentrations of 25 and 50 nM, the unspecific effects associated with the scramble oligonucleotide are, most likely, a result of an unspecific interaction of the scramble oligonucleotide with miR-21 and not a result of the transfection process.

Concerning the mRNA levels of the two major cytokines IL-6 and IL-1β, we were able to observe significant differences in the IL-6 mRNA levels in cells transfected with anti-miR-21 oligonucleotide and scramble oligonucleotide, at an oligonucleotide final concentration of 100 nM. However, no significant differences in the IL-6 mRNA levels were detected when the cells were activated with LPS (Fig.26b). With respect to IL-1β mRNA no differences in the mRNA levels of this cytokine were observed, neither in the resting state, nor in an LPS-induced activated state (Fig.26c).

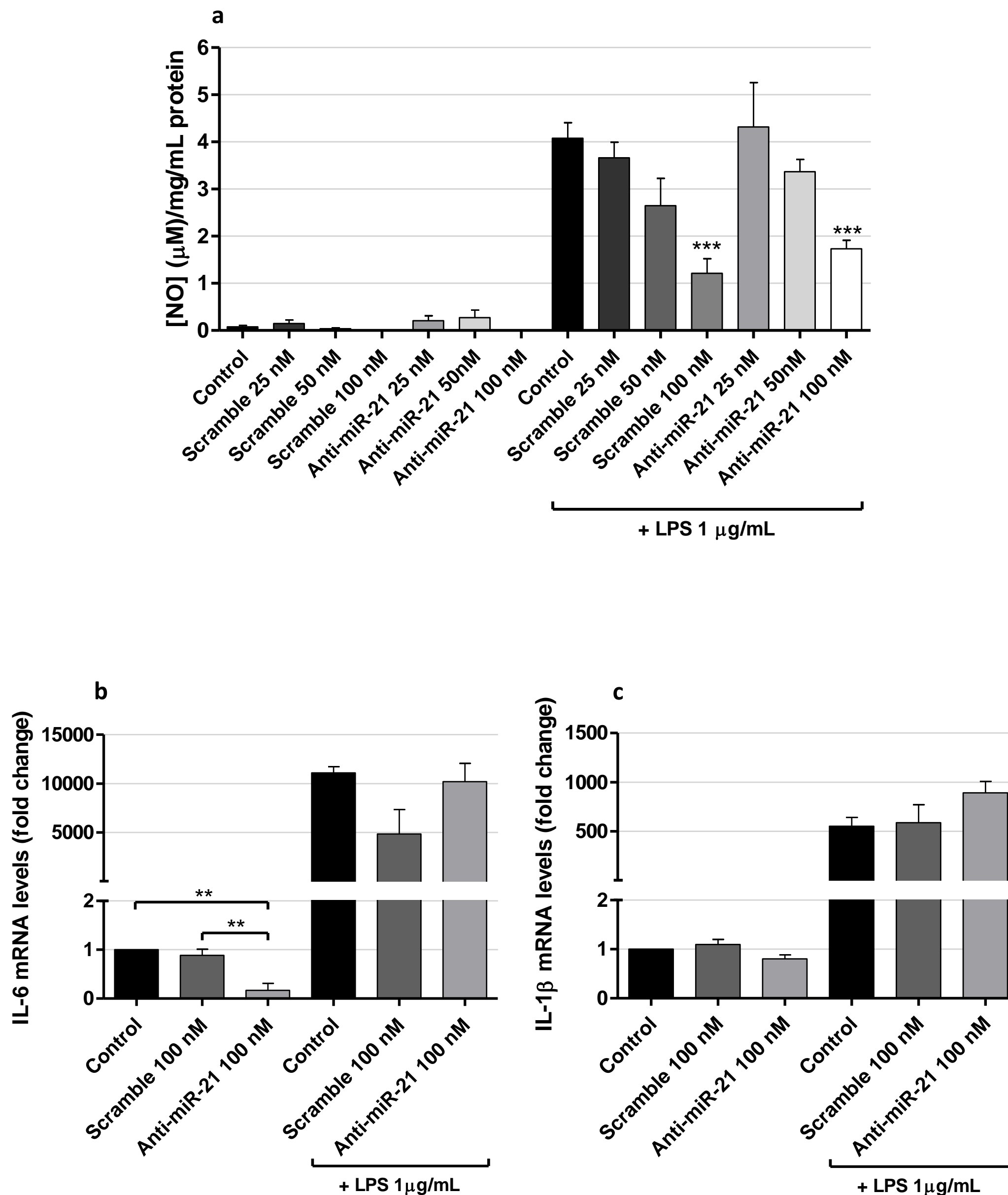

Fig. 26 – Assessment of microglia-mediated imune response following miR-21 silencing via DLS lipoplex. **(a)** – Twenty-four hours after plating, N9 cells were transfected with DLS lipoplexes at oligonucleotide final concentrations of 25, 50 or 100 nM. Twenty-four hours after transfection, cells were incubated for 24h in the absence or presence of 1 μg/mL of LPS. Nitric oxide levels were assessed using the Griess test and the NO concentrations obtained were normalized with the total protein levels of each sample; **(b)** and **(c)** – IL-6 (b) and IL-1β (c) mRNA levels were determined in both resting and activated conditions by q-PCR. Q-PCR results are presented as mRNA fold change with respect to resting N9 untransfected cells; Results are representative of four independent experiments. *ns – non-significant, * – P < 0.05, ** – P < 0.01, *** – P < 0.001.*

4.3 – MiR-21 silencing in microglia promotes glioma cell death

With the aim of understanding how miR-21 silencing in N9 microglia cells could affect their response to the glioma cells, we incubated GL261 cells (a mouse glioma cell line) with conditioned medium derived from either resting or LPS-activated wild type, GFP and 21 N9 cells, for 48h. We then assessed the extent of GL261 cell death, through flow cytometry analysis using Annexin-V-PE and 7-aminoactinomycin D (7-AAD) labeling, and caspase 3/7 activation using a fluorescence assay. Annexin-V specifically binds to phosphatidylserine (PS) residues, which are translocated from the inner leaflet to the outer leaflet of the cell membrane during apoptosis, thus effectively labeling apoptotic cells. On the other hand, 7-AAD is a fluorescent chemical compound which intercalates with DNA. This compound is only able to reach the DNA when membrane integrity is compromised, thus constituting a good indicator of necrosis. Double labeled cells (both Annexin-V and 7-AAD positive cells) are considered as late-apoptotic.

As observed in Fig.27, exposure of GL261 cells to conditioned medium derived from LPS-stimulated N9 21 cells resulted in an overall increase of cell death (particularly in the late apoptosis; Fig.27a), and in a significant increase in caspase 3/7 activation (Fig.27b). Interestingly, the conditioned media derived from wild type and GFP N9 cells seem to increase cell viability with respect to that observed in control conditions. Furthermore, these same conditioned mediums do not significantly induce caspase 3/7 activation. Therefore, taken together, these results suggest that miR-21 silencing in microglia sensitizes these immune cells towards a more effective immune response to glioma cancer cells.

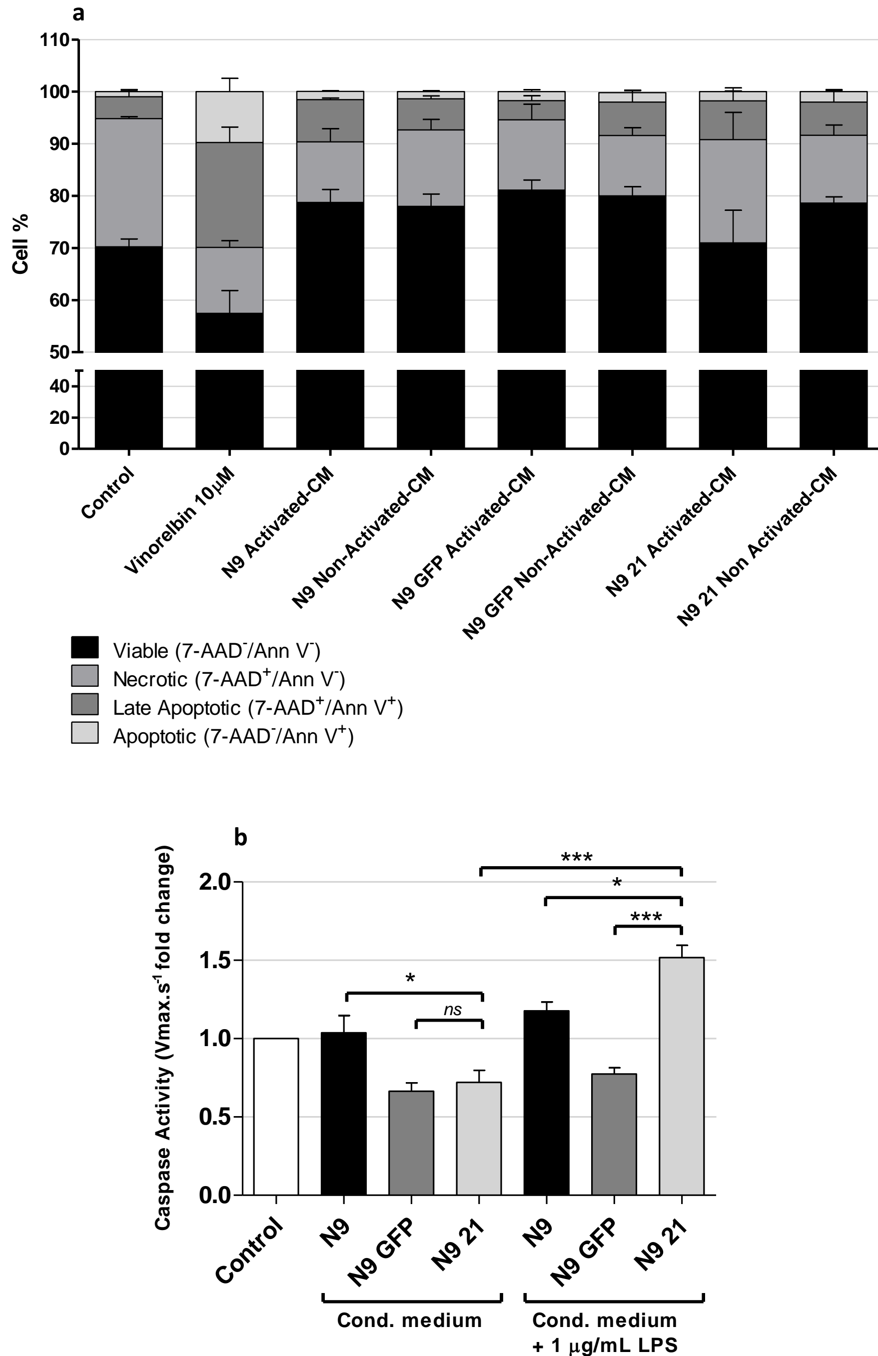

Fig. 27 – Assessment of the extent of glioma cell death after incubation with microglia conditioned medium. GL261 cells were plated 24h before incubation with microglia conditioned medium derived from either resting (non-activated-CM) or wild type, GFP and 21 N9 cells stimulated with 1 µg/mL of LPS (activated-CM). **a** – After incubation, cells were labeled with Annexin-V-PE and 7-AAD and analyzed by flow cytometry; 10.000 events were collected per sample. As a positive control, cells were incubated with 10 µM of Vinorelbine.; **b** – After incubation with the conditioned media, cells were collected and lysed and caspase 3/7 substrate was then added to the supernatant. Caspase 3/7 activity was analysed by fluorescence spectroscopy and results are presented as fold change with respect to control (untreated cells). Results are representative of three independent experiments. *ns – non-significant; * – P < 0.05; *** – P < 0.001.*

5 – Discussion

Over the past few years the interest in understanding microglia-mediated immune response to glioma tumors has increased, and the ability of glioma cells to modulate microglia-mediated immune response has been a matter for debate. Clinical observations and *in vitro* studies indicate that microglia and macrophages can infiltrate the brain tumor tissue in high numbers and may play a supportive role during tumor development through the expression of matrix metalloproteinases that lead to tumor growth and infiltration [64]. Several studies also suggest that the glioma microenvironment can modulate the phenotype of infiltrating microglia and macrophages, causing an impairment or complete ablation of pro-inflammatory cytokine production [48, 49, 67].

In the present study, we observed that glioma conditioned medium derived from GL261 cells (mouse glioma cell line) induced a significant expression of two pro-inflammatory cytokines IL-1β and TNF-α (Fig.14a and b) and a slight decrease of SOCS-1 (inhibitor of cytokine signaling), suggesting that microglia activation is not completely impaired in these cells. A slight increase in the levels of miR-155 (Fig. 16b), which is known for being a pro-inflammatory miRNA, as well as a significant increase in the mRNA levels of iNOS, an enzyme responsible for the production of NO and a potent pro-inflammatory mediator. Interestingly, despite the significant enhancement of iNOS mRNA levels by glioma conditioned medium, no NO production was detected. However, when the cells, previously exposed to glioma conditioned medium, were incubated with LPS, NO production was significantly higher with respect to NO production in cells incubated with LPS alone. Under these conditions, an increase in the mRNA levels of iNOS was also observed (Fig.15). Finally, a significant increase in the levels of miR-21 was detected, a miRNA reported as being involved in the regulation of immune response [83], although the role of this miRNA in such process remains unclear. Inthis regard, contradictory data have been reported in the literature in the literature, suggesting either a pro [130] or an anti-inflammatory [106] role for this miRNA.

Our results are in agreement with those obtained by Kees and colleagues, which revealed that microglia isolated from gliomas supported tumor cell growth, migration, and invasion [131]. However, the authors have shown that upon stimulation of tumor isolated microglia with poly (I:C) (a TLR-3 agonist), microglia secreted factors that exerted toxic and suppressive effects on glioma cell lines. Another study performed by Yeh and colleagues reported that glioma cells stimulate microglia to produce pro-inflammatory cytokines, which are then used by glioma cells to enhance their migration ability [132].

Taken together, our results suggest that microglia does not respond strongly to glioma-conditioned medium. However, when microglia cells are stimulated with stronger activation stimuli, such as LPS, followed by incubation with glioma conditioned medium, an enhancement in the immune response is observed. These findings suggest that under these conditions, microglia is induced to a "primed state" in its immune response and both miR-155 and miR-21 may play a role in this priming process.

The results presented in Fig. 16 show that miR-21 and miR-155 were overexpressed in N9 microglia cells following incubation with glioma conditioned medium derived from GL261 cells. Since miR-155 has already been described by us and others as being a pro-inflammatory miRNA, responsible for the regulation of microglia mediated immune response through SOCS-1 targeting [120, 121], attention on miR-21 was focused and how the silencing of this miRNA could affect microglia immune response.

In order to answer this question we silenced miR-21 in N9 microglia cells using two different strategies: lentiviral transduction and DLS lipoplex transfection. Our results have shown that miR-21 silencing in microglia strongly affected its ability to produce NO upon LPS stimulation and significantly decreased the levels of the pro-inflammatory cytokine IL-6 when the cells were in the resting state (Fig. 19a and b). On the other hand, IL-1β levels were not significantly affected by miR-21 silencing (Fig.19c). Our results corroborate the work performed by Weber and colleagues, in which the authors reported that miR-21 expression is directly proportional to NO production. In their study, performed in endothelial cells, the authors observed that miR-21 silencing reduced eNOS phosphorylation and NO production, while miR-21 overexpression increased both eNOS phosphorylation and NO production [133]. Since PTEN has been reported has being a direct target of miR-21 and shown to negatively regulate eNOS phosphorylation and NO production, through the antagonism of the PI3K/Akt pathway [134], a link can be proposed between miR-21, PTEN expression and NO production, where miR-21 overexpression suppresses PTEN expression and increases both Akt and eNOS phosphorylation, resulting in an increase of NO production [133].

In another recent study performed by Okayama *et al.* it was reported that miR-21 induction is dependent on iNOS through $KRAS^{G12D}$ activation [135]. $KRAS^{G12D}$ is a mutated form of the GTPase Ras protein which has been associated to enhance cell proliferation and tumorigenesis [136]. The authors based their assumptions on another previous study where it was described that Ras activation increases the expression of miR-21, and in turn, miR-21 enhances tumor proliferation and survival by targeting both antagonists of Ras signaling

pathways and proapoptotic genes [137]. In their own study, Okayama and colleagues, silenced iNOS in $KRAS^{G12D}$ transgenic mice and investigated miR-21 expression in both iNOS wild type and iNOS silenced $KRAS^{G12D}$ transgenic mice. They observed that iNOS deficiency alone did not alter miR-21 expression without KRAS activation, but significantly reduced miR-21 expression when KRAS was activated. From these results they suggest that iNOS may alter miR-21 expression depending on the activation of KRAS. Given that miR-21 is a downstream target of Ras, the authors propose a regulation of miR-21 expression by iNOS-derived NO [135]. Although we did not check iNOS levels in microglia cells with silenced miR-21, taken together with the results reported by Weber and by Okayama, our data suggest that iNOS levels and miR-21 expression are mutually dependent, and a regulatory feedback loop may exist involving iNOS, KRAS and miR-21, which will affect NO production.

Regarding PDCD4, IL-6 and miR-21, in a study performed by Sheedy *et al.*, it is suggested that PDCD4, is required for IL-6 expression [106]. Our results, have shown that lentiviral mediated miR-21 silencing in N9 microglia cells (N9 21 cells) led to an increase in PDCD4 mRNA and protein levels when the cells were in a resting state (Fig.20). However, contrary to the results of Sheedy and colleagues, miR-21 silencing resulted in a significantly decrease in the levels of this cytokine with respect to lentiviral-transduced control cells (N9 GFP cells; Fig.19b). Moreover, when both cell lines (N9 21 and N9 GFP) were stimulated with LPS, PDCD4 mRNA levels were significantly higher in N9 21 cells as compared to N9 GFP cells (Fig.20). On the other hand, no significant differences could be observed in IL-6 levels between the two types of cells, although a small decrease in the amount of IL-6 could be detected in N9 21 activated cells. Furthermore, following DLS lipoplex-mediated miR-21 silencing, an even larger difference was observed in the IL-6 levels of resting N9 cells transfected with anti-miR-21 oligonucleotides with respect to both scramble-transfected and untransfected cells (Fig.26). However, we were not able to observe the same tendency as that detected in the lentiviral transduced cells, when the cells were stimulated with LPS following DLS-mediated miR-21 silencing, probably due to the fact that the scramble oligonucleotide interacts inespecificaly with miR-21 (as shown in Fig.24). Overall, our results indicate that IL-6 expression is partially dependent on miR-21 levels, but in a PDCD4 independent manner. In addition, other studies have suggested that miR-21 is induced by IL-6 overexpression in a STAT-3 dependent way [125, 138]. Taken into consideration all these findings we suggest that miR-21 and IL-6 levels are possibly dependent from each other through a mechanism that, although not completely clear, seems to be independent of PDCD4 expression.

As previously discussed, glioma cells induce a slight immune response in microglia, increasing the levels of the cytokines IL-1β and TNF-α, significantly inducing the expression of iNOS mRNA, and inducing the expression of both miR-21 and miR-155. Surprisingly, an increase in glioma cell viability was observed, when glioma cells were exposed to conditioned medium derived from resting or LPS-activated wild type, GFP or 21 N9 microglia cells, with respect to control cells (untreated cells) (Fig.27a). These results indicate that this immune response is not efficient in promoting tumor cell death, which is in agreement with the results obtained by other groups [64, 66]. Interestingly, the only exception observed to this tendency regards the cells exposed to conditioned medium derived from LPS-activated N9 21 cells. Under these conditions, we observed an increase in late apoptotic cells with respect to control cells (Fig.27a). Moreover, when (Fig.27b) glioma cells were incubated with conditioned media derived from wild type and GFP N9 cells no significant change in the activity of caspase 3/7 was obtained. However, the medium derived from activated N9 21 cells, significantly induced caspase 3/7 activity in glioma cells. Taken together, these results suggest that miR-21 silencing in microglia may affect the ability of these cells to respond to glioma cells.

In a study performed by Frei and colleagues, it was reported that FasL, a ligand of FasR (also known as CD95/APO-1) which is a cell surface receptor and member of the tumor necrosis factor receptor superfamily implicated in apoptosis, was able to induce apoptosis in several glioma tumor cell cultures isolated from human patients [139]. Another study performed by Rubinchik *et al.*, showed that FasL, in combination with TNF-related apoptosis-inducing ligand (TRAIL), enhanced apoptosis in glioma cell lines [140]. Interestingly, FasL has been reported as a direct target of miR-21 [141], and, therefore, an enhanced expression of FasL in N9 21 cells might be a plausible explanation for the results observed in glioma cell viability and caspase 3/7 induction after incubation with the conditioned medium derived from N9 21 activated cells.

6 – Concluding remarks and future perspectives

6 – Concluding remarks and future perspectives

The results obtained in this work led to several interesting conclusions.

- Glioma-conditioned medium leads to priming of microglia-mediated immune response

From the results observed when microglia is exposed to conditioned medium derived from glioma cells, we can suggest that microglia does not strongly respond to glioma cells. However, an enhancement in microglia-mediated immune response was observed when microglia cells, previously exposed to glioma conditioned medium,are submitted to a strong stimulus. These findings suggest that microglia might be primed by glioma cells to strongly respond to a pro-inflammatory stimulus. Moreover, the induction of miR-21 and miR-155 expression in microglia cells following exposure to glioma conditioned medium suggests that both these miRNA may play a role in this priming process.

- Silencing of miR-21 affects microglia-mediated innate immune response

From the results observed on the decreased NO production and expression of IL-6 in resting microglia cells, previously transduced/transfected to silence miR-21, we can suggest that miR-21 plays an important role in microglia-mediated immune response. Although these effects were abolished when cells were stimulated with LPS, a tendency towards a lower expression of IL-6 with respect to control cells was observed. However, miR-21 silencing did not seem to influence the expression of IL-1β, TNF-α or IFN-β (data not shown), indicating that these particular inflammatory mediators are not under the influence of this miRNA.

- MiR-21 silencing in microglia increases cell glioma cell death

From the results obtained on the increase of glioma cell death and caspase 3/7 activation upon incubation with microglia-derived conditioned medium, we can suggest that miR-21 is important during microglia-mediated response to glioma tumor cells. It would be interesting to evaluate a possible role of FasL signaling in this process, since this ligand has been reported not only to enhance glioma cell apoptosis, but also to be a direct target of miR-21.

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