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ROLE OF B7-H3/CD276 IMMUNE CHECKPOINT IN GLIOBLASTOMA

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Résumé

Le glioblastome (GBM) est le cancer du cerveau le plus agressif et le plus mortel. Ce gliome de grade 4 est caractérisé par une récurrence quasi-inévitable et est difficile à traiter. Les thérapies disponibles sont une résection totale de la tumeur via chirurgie, et une combinaison de la radiothérapie et de la chimiothérapie avec des agents alkylants comme le temozolomide. Ces thérapies ne permettent malheureusement pas la guérison du patient mais seulement d'augmenter la durée de vie du patient de quelques mois, atteignant ainsi en moyenne 15 mois de survie après le diagnostic. De nombreux types de thérapies sont en cours d'investigation, avec un grand nombre de cibles thérapeutiques à explorer. Une des thérapies les plus en essor pour le moment contre le cancer est l'immunothérapie, visant à stimuler la réponse immunitaire contre les cellules tumorales. Les cibles de ce type de thérapie sont, entre autres, des points de contrôle (checkpoints) immunitaire dont l'activation va le plus souvent à l'encontre d'une réponse immunitaire. Dans beaucoup de cancers, ces checkpoints sont surexprimés et empêchent alors une réponse destructive du système immunitaire contre les cellules cancéreuses.

Dans ce travail, nous nous sommes intéressés à une protéine transmembranaire de la famille des super immunoglobulines B7, appelée B7-H3 et codée par le gène *CD276*. Cette protéine a été identifiée comme étant exprimée dans de nombreux cancers et ayant un rôle dans la progression tumorale via différents mécanismes, qui sont aujourd'hui fortement controversés. Nos résultats préliminaires ont démontré l'expression de B7-H3 dans le GBM, et nous avons donc voulu identifier son rôle précis.

Nos résultats ont confirmé l'expression de B7-H3 dans les lignées cellulaires classiques de GBM ainsi que dans des cellules dérivées de patients (*in vitro* et *in vivo*). Plusieurs expériences ont été effectuées dans le but de clarifier le rôle de cette protéine dans (1) la croissance tumorale et dans (2) la régulation des cellules immunitaires. En utilisant un modèle *knock-out* de B7-H3, nous avons confirmé que B7-H3 n'était pas requise pour la croissance tumorale dans un modèle immunodéficient. Par contre, les résultats obtenus n'ont pas permis de démontrer le rôle de B7-H3 dans la régulation directe immune lymphocytaire. De plus amples investigations sont nécessaires pour mieux comprendre le rôle de cette protéine dans le GBM et l'exploiter dans des approches de thérapie.

Abstract

Glioblastoma (GBM) is the most aggressive and lethal brain cancer. This grade 4 glioma is characterized by systematic recurrence and is really difficult to treat. Available therapies are complete resection followed by radiotherapy and chemotherapy with temozolomide (an alkylating agent). These therapies are not sufficiently effective and allow only a modest improvement in life expectancy to reach about average 15 months of survival from the diagnosis. Many therapies are under investigation based on a wide range of therapeutic targets to explore. One of the therapies that are most investigated today for cancer is immunotherapy. This aims to boost the immune response against tumor cells. Targets for this type of therapy are numerous and are principally part of the immune system, such as immune checkpoint proteins that are often acting on immune cells for shutting down the immune response. In many cancers, those checkpoints are overexpressed and prevent a destructive immune response against cancer cells.

In this work, we focused on a transmembrane protein from the B7 super-immunoglobulin family, B7-H3, also called CD276. This protein has been identified to be overexpressed in many cancers and plays a role in tumor progression through different mechanisms. All these mechanisms of action are highly debated. Preliminary work has demonstrated that B7-H3 is expressed in GBM, and we wanted to identify its role on tumor growth.

Our results confirmed B7-H3 expression in classical GBM cell lines and in patient-derived glioblastoma stem-like cells (*in vitro* and *in vivo*). From that step, different experiments have been conducted for the purpose of determining the role of B7-H3 on (1) tumor proliferation and growth, and (2) immune cell activation. Using a knock-out model for B7-H3, we showed that this protein was not required for sustaining tumor growth in an immunodeficient mouse model. On the other hand, the results we obtained could not allow us to address the role of B7-H3 on direct lymphocyte immune cell regulation. Further investigation is required for understanding the determinants of B7-H3 function in glioblastoma and harness it in immunotherapeutic approaches.

Abbreviations

2-HG	2-Hydroxy-glutarate
Ab	Antibody
ADC	Antibody Drug Conjugate
ALT	Alternative Lengthening Telomeres
APC	Antigen Presenting Cell
ATRX	Alpha-Thalassemia X-linked Retardation
B7-H3	B7 Homolog 3
BBB	Blood Brain Barrier
BM-TAM	Bone-Marrow-derived Tumor Associated Macrophages
BSA	Bovine Serum Albumin
CAR T cells	Chimeric Antigen Receptor T cells
CDKN2A/B	Cyclin-Dependent Kinase Inhibitor 2 A/B
CNA	Copy Number Alteration
CNS	Central Nervous System
CSF	Cerebro-Spinal fluid
DC	Dendritic Cell
DNA	Desoxyribonucleic Acid
EGFR	Epithelial Growth Factor
EGFRvIII	Epithelial Growth Factor variant III
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fv	Variable Fragment
GAM	Glioma-Associated Macrophages
GBM	Glioblastoma
GSCs	Glioblastoma Stem-like Cells
HRP	Horseradish Peroxidase
IDH	Isocitrate Dehydrogenase
KO	Knock-Out
LGG	Low Grade Glioma
M1	Microglia/Macrophages type 1
M2	Microglia/Macrophages type 2
mAb	Monoclonal Antibody

MAP kinase	Mitogen Associated Protein kinase
MFI	Mean Fluorescence Intensity
MGMT	O^6-MethylGuanine-DNA-MethylTransferase
MG-TAM	Microglia-derived Tumor Associated Macrophages
NDS	Normal Donkey Serum
NK	Natural Killer
NT_2	Non-Target cocktail 2
OCT	Optimal Cutting Temperature compound
OE	Over-Expressed
ON	Over Night
PBS	Phosphate-Buffered Saline
PDGFR	Platelet-Derived Growth Factor Receptor
PFA	Paraformaldehyde
RNA	Ribonucleic Acid
RT	Room temperature
SVZ	Subventricular Zone
TAA	Tumor Associated Antigen
TAM	Tumor Associated Macrophages
TCR	T Cell Receptor
TERT	Telomerase Reverse Transcriptase
Th1	T Helper Cell Type 1
Th2	T Helper Cell Type 2
TIL	Tumor Infiltrating Lymphocytes
TLT2	TREM-Like Transcript 2
TME	Tumor Microenvironment
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
TP53	Tumor Protein 53
Treg	T lymphocytes regulator
TREM2	Triggering Receptor Expressed on Myeloid cells 2
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

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Introduction

1. WHO classification of Central Nervous System tumors

The World Health Organization (*WHO*) classification of Central Nervous System (*CNS*) tumors was first based on anatomopathological features of the tumor but was not sufficient to reliably predict patient clinical outcome or treatment response. The improvement of diagnosis technologies such as immunostaining, molecular genetics/profiling, Desoxyribonucleic Acid (*DNA*) sequencing, methylome profiling, etc. increased our knowledge in the molecular profile of CNS tumors, which proved to allow a more precise classification. The identification of these molecular alterations leads to an association of morphological patterns and genetic characteristics, that are now needed for an "integrated" diagnosis. Many types of CNS tumors exist, with different molecular patterns and morphological characteristics. In this work, we will focus on the global classification of gliomas (Louis et al., 2021).

2. Gliomas

Gliomas are the most frequent primary brain tumors, arising from glial cells (Ostrom et al., 2014). Gliomas are classified into different types: adult-type diffuse gliomas, pediatric type diffuse low grade gliomas, pediatric-type diffuse high grade gliomas and circumscribed astrocytic gliomas. I will here focus on adult-type diffuse gliomas, that are categorized in subtypes according to histological features and molecular alterations. These genetic modifications are multiple, but some are observed more frequently and are therefore considered as biomarkers for the different subtypes of adult-type diffuse gliomas (**Figure 1**). An integrated diagnosis is thus possible, taking into account histology (diffuse astrocytic and oligodendroglial gliomas), molecular features and other key information (R. Chen et al., 2017; Louis et al., 2021).

Adult type diffuse gliomas
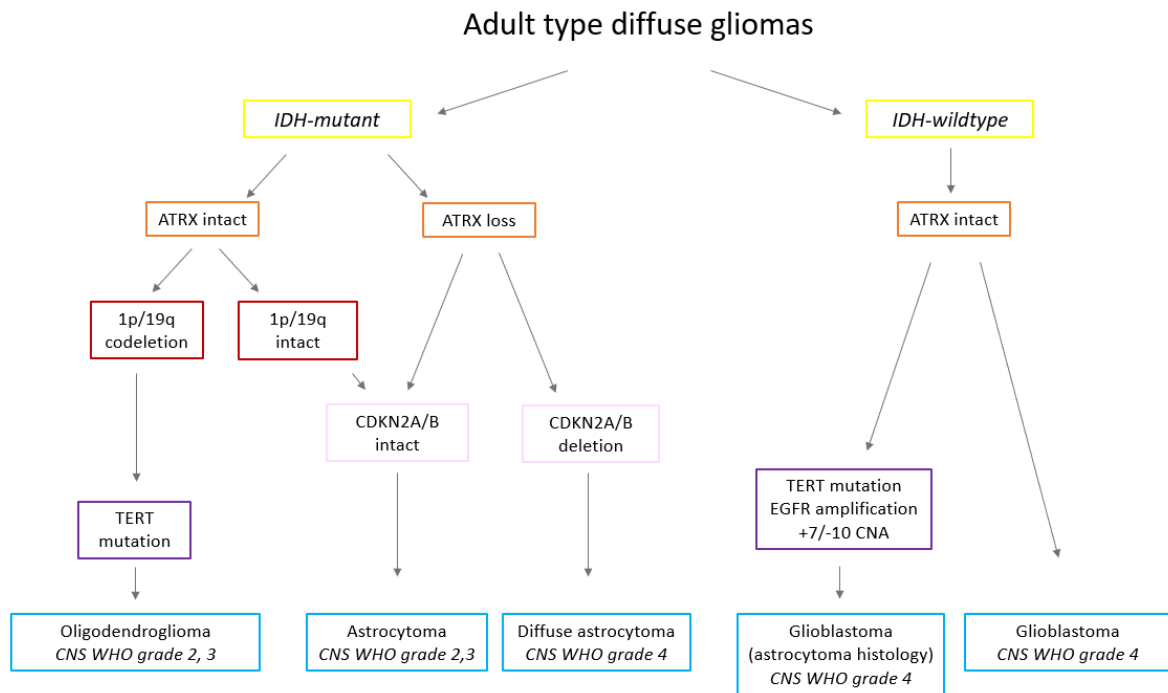
Figure 1. 2021 CNS WHO classification of adult type diffuse gliomas according to different level of molecular classifiers: Isocitrate Dehydrogenase (*IDH*) coding gene as the first level, ATRX as the second level, 1p/19q-codeletion, Cyclin-Dependent Kinase Inhibitor 2 (*CDKN2A/B*) and Telomerase Reverse Transcriptase (*TERT*), Epithelial Growth Factor Receptor (*EGFR*), Epithelial Growth Factor Receptor variant III (*EGFRvIII*), +7/-10 somatic Copy Number Alteration (*CNA*) as the following levels.

2.2. Molecular characterization

2.2.1. IDH mutation

IDH mutation is the first level of molecular classifier for adult diffuse gliomas. Isocitrate dehydrogenase (*IDH*) exists into two isoforms, IDH1 and IDH2. It is an enzyme of Krebs cycle. IDH catalyzes the oxidative decarboxylation of isocitrate producing alpha-ketoglutarate. When mutated, IDH is not catalyzing the production of alpha ketoglutarate but rather increasing 2-hydroxyglutarate (*2-HG*) production from isocitrate. 2-HG is an oncometabolite, and its accumulation changes DNA and histone methylation, providing a G-CIMP or CpG island phenotype and allowing the expression of different genes involved in tumor progression. The most frequent mutation concerns isoform IDH1, modifying arginine in position 132 into histidine (IDH1 R132H) (Ohgaki & Kleihues, 2013; Wirsching et al., 2016). Glioblastoma is characterized by an IDH-*wildtype* phenotype which is a sign of a poor prognosis, while IDH-*mutant* phenotype is associated with lower-grade gliomas and indicates a more favorable outcome.

2.2.2. Alpha-Thalassemia X-linked Retardation syndrome (ATRX)

ATRX is a chromatin remodeler. A mutation of this protein also exists in absence of this retardation syndrome. The loss of ATRX can be due to mutations, deletions, or fusions with other genes. For example, it is often associated with Tumor Protein 53 (*TP53*) or Alternative Lenghtening Telomeres (*ALT*) phenotype that are met in different tumors (Wirsching et al., 2016; Reinhardt et al., 2018). The absence of ATRX is linked to damages in DNA. This instability leads to the activation of the ALT phenotype that leads to telomeres elongation without the need of telomerase. This parameter is associated with a lower mean survival rate (Haase et al., 2018).

2.2.3. 1p/19q codeletion

This mutation is due to a translocation between the short arm from chromosome 1 and the long arm from chromosome 19, resulting in the loss of the two arms. It corresponds to the second level of molecular classification and is often a genetic marker that predicts a good survival. 1p/19q codeletion is always found in oligodendroglioma (IDH mutant), but not in astrocytomas neither in IDH-wildtype tumors (Ohgaki & Kleihues, 2013; Nuechterlein et al., 2021).

2.2.4. Cyclin-Dependent Kinase Inhibitor 2 (CDKN2A/B)

CDKN2A and B genes are coding for 2 and 1 tumor suppressor protein respectively. CDKN2A encodes for p14^{ARF} protein, acting indirectly on p53 and pRb by enhancing their activity in cell cycle arrest and for p16^{INK4a} that binds cyclin-dependent kinase slowing its activity. p15^{INK4b} is coded by CDKN2B and plays the same role as p16. The involvement of CDKN2A/B plays an important role in tumor development in general. Deletion of these genes leads to a lack of cell cycle inhibition. Such a constitutive activation allows the cell to proliferate in an uncontrolled way. CDKN2A/B is newly recognized as a new entity in the 2021 WHO classification to distinguish IDH-*mutant* tumors. In point of fact, IDH-*mutant* phenotype encompasses either Low Grade Gliomas (*LGG*) and higher grade astrocytomas. Even if CDKN2A/B might be present in glioblastoma, it is found in WHO grade 4 diffuse astrocytoma,

making this mutation an independent prognosis factor associated with a poor survival for IDH-*mutant* astrocytomas (Reinhardt et al., 2018; Ma et al., 2020; Huang, 2022).

2.2.5. *Telomerase Reverse Transcriptase (TERT)*

TERT is an enzyme that serves for telomerase maintenance and is present only in cells able of high proliferation, such as tumor cells or stem cells. Indeed, in these cells only, telomere length is maintained by telomerase. TERT undergoes a mutation in its promoter and is thus able to add repetitive sequence of nucleotides at the end of DNA sequence (Omuro & DeAngelis, 2013; Wirsching et al., 2016; Louis et al., 2021).

2.2.6. *Epithelial Growth Factor Receptor (EGFR) or EGFR variant III amplification*

EGFR is present on cell surface and is considered as the most prevalent genetic alteration in glioblastoma. Two mutations are possible: EGFR is amplified, more expressed and more active, or an activating mutation corresponding to a deletion in the ligand-binding domain leads to a splicing variant called EGFR variant III (*EGFRvIII*) which is constitutively active (Wirsching et al., 2016). This variant III induces cell transformation via different pathways enhancing signals involved in glial cell proliferation, angiogenesis, inflammation, inhibition of apoptosis and stem cell renewal (Akbari et al., 2018).

2.2.7. *+7/-10 copy number variation (CNA)*

This somatic copy number alteration consists in a combination of a total gain of chromosome 7 and a loss of chromosome 10 (Louis et al., 2021). CNA has been shown to allow making diagnosis before having any idea of the molecular status of the glioblastoma. +7/-10 copy number alteration is only present in IDH-*wildtype* gliomas and is thus a diagnosis-criteria for glioblastoma (Nuechterlein et al., 2021).

3. Glioblastoma

As previously said, Glioblastoma is the most common and lethal brain tumor of the CNS. This grade 4 adult type diffuse glioma is often characterized by an IDH-*wildtype* status. This tumor is either primary or arises from LGG with an IDH-*mutant* status (Wirsching et al., 2016). It develops rapidly in elderly patients, between 60 to 80 years and is more observed in men than women. There is no known cause for this tumor. Many different factors either intrinsic or environmental have been possibly identified to play a role in the onset of such tumors (Omuro & DeAngelis, 2013). In 5% of GBM cases, genetics increase the risks. Glioblastoma is a tumor that is highly invasive and infiltrative, meaning that GBM cells are able to move from the initial tumor site to other parts of the brain. GBM is characterized by systematic recurrence (Alexander & Cloughesy, 2017).

This brain tumor can either develop directly into a grade 4 glioblastoma or arise from an existing LGG, called respectively primary or secondary GBM. The difference in tumor origin shows a wide range of mutations that are used for diagnosis (Ohgaki & Kleihues, 2013). GBM is also characterized by high heterogeneity, which is both intertumoral, showing important differences between patients (**Figure 2**) and intratumoral, since different subpopulations of cells have been observed within one single tumor. Among different patients, we observe differences in morphology, genetics, glioblastoma cell markers etc. These heterogenous cell populations are responsible for resistance to therapies, that target only specific subpopulations (Skaga et al., 2019; Ou et al., 2021). Because of this tumor cell recalcitrance, either at primary tumor site or a little further into the brain, treating glioblastoma is a real challenge (Campos et al., 2016). To be able to treat GBM and prevent its recurrence form to reappear, a solution is to identify different targets to kill specifically GBM cells, combining them to reach as many cells as possible.
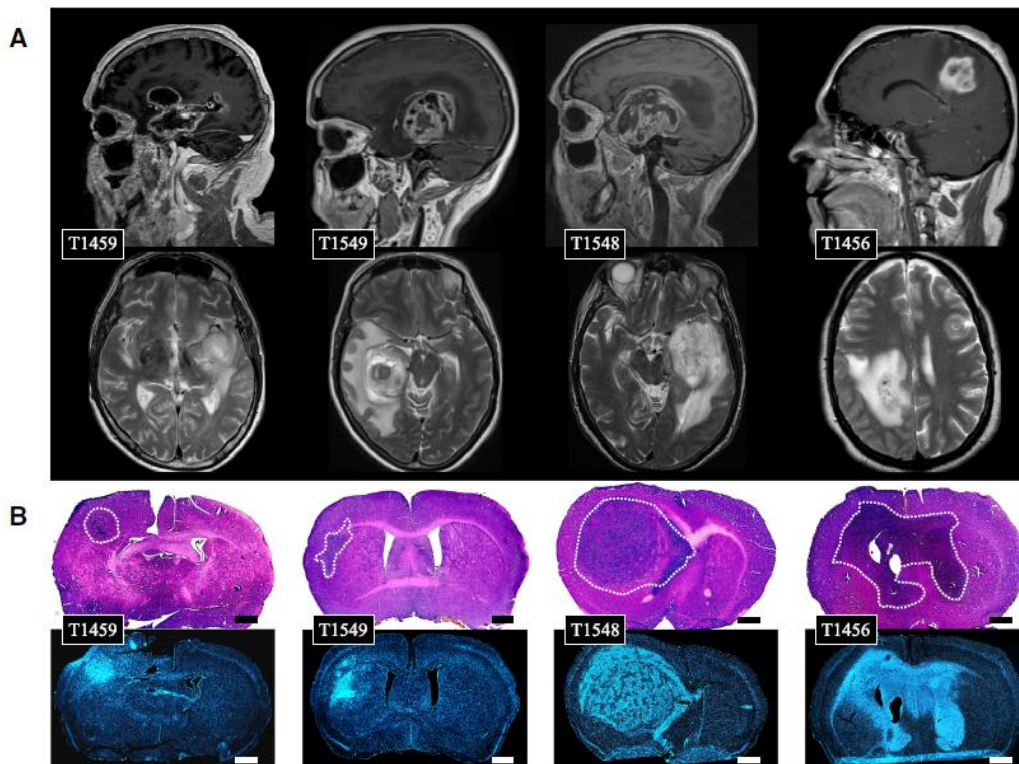
Figure 2. Magnetic Resonance Imaging (*MRI*) of a cohort study of four GBMs. (A) These four patient-derived Glioblastoma Stem-like Cells (GSCs) were injected in mice brains by stereotactic injection. (B) After mice sacrifice, immunostaining via Hematoxylin-Eosin demonstrated the heterogeneity between the four GBM cell population behavior *in vivo*. The xenografts images correspond to the location heterogeneity in human brain (Skaga et al., 2019).

3.1.   The cell of origin

The cell of origin that gives rise to GBM is not known. However, we can consider that a subpopulation of GBM cells with stem cells characteristics has been identified. We call them GBM Stem-like Cells (*GSCs*). These cells are endowed with self-renewing, tumor initiation, proliferation and migration abilities. These GSCs share many characteristics with neural stem cells but it does not imply that neural stem cells are cells giving rise to GBM. Different models are currently discussed for explaining GSC development and maintenance. GBM cells can acquire a stem cell phenotype in some conditions (stochastic, plastic model), but they could also arise from a pool of neural stem and/or progenitor cells located in SVZ (*subventricular zone*) (hierarchical model) (Wirsching et al., 2016). The SVZ is the largest niche of neurogenesis in the adult CNS. A second neurogenic niche is in the hippocampal *dentate gyrus*. Neural stem cells that are in these niches could undergo mutations, which could make them able to evade

these neurogenic zones and proliferate in an uncontrolled manner, inducing tumorigenesis (Khalifa et al., 2017; J. H. Lee et al., 2018). Different cells of origin have been suggested for different types of gliomas (Suvà & Tirosh, 2020). The heterogeneity of the cells of origin could also play an important role in the intratumoral heterogeneity and takes a great part in the therapeutic challenge (Ou et al., 2021).

3.2. Histology and molecular features

Histologically, GBM is usually characterized by different parameters. Observing GBM tissue reveals an hypercellularity corresponding to an important mitotic activity, forming a pseudopalisade surrounding necrosis zones that are present (**Figure 3**). The occurrence of blood vessels in the tumor mass confers to GBM a highly invasive and infiltrative capability (Omuro & DeAngelis, 2013; Alexander & Cloughesy, 2017; Akbari et al., 2018). As told earlier, GBM can be divided in primary and secondary tumor. This difference in tumor origin makes them two distinct entities that are classified according to key mutations by WHO 2021 classification. Secondary glioblastoma includes mutations from lower grade gliomas as it develops from existing tumor, whereas primary glioblastoma shows mutations associated with poorer prognosis. Some of these mutations are specific of glioblastoma, but not considered as gliomas classifiers. However, their presence may justify therapy resistance (Faleh Tamini et al., 2017). These mutations are for example present in the genes encoding for MGMT, PDGFR and VEGF/VEGFR.
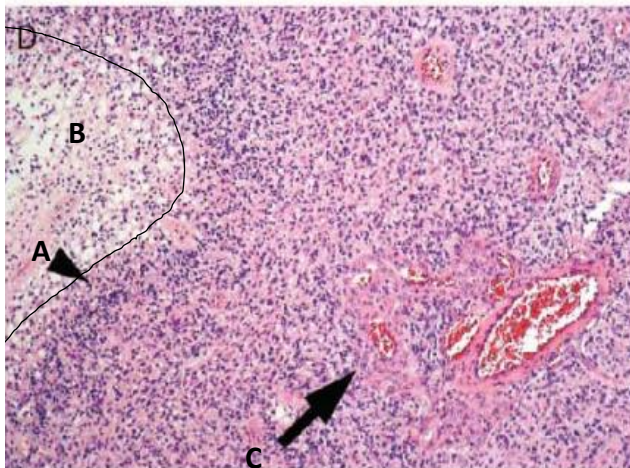
Figure 3. Global hypercellularity in the tumor. Mitotic cells are forming a pseudopalisade (A) around a lighter necrosis zone (B). Microvascular hyperplasia, formation of microvessels (Rong et al., 2006). (C)

3.2.1. O^6-MethylGuanine-DNA-MethylTransferase (MGMT)

MGMT is an enzyme responsible for direct DNA repair. The promoter of this enzyme is often hypermethylated in primary GBM, which prevents its expression (Butler et al., 2020). The hypermethylation of the *MGMT* promoter allows a certain sensitivity to chemotherapy with temozolomide (*TMZ*) alkylating agent, which induces DNA damages that are therefore not efficiently repaired. In recurrent GBM, there is no MGMT promoter methylation suggesting a rearrangement in MGMT expression. This higher expression of MGMT in recurrence leads to a better DNA repair activity, repairing damages caused by the TMZ and acquiring resistance to chemotherapy (Hegi et al., 2019; Oldrini et al., 2020).

3.2.2. Platelet-Derived Growth Factor Receptor (PDGFR)

Another mutation that characterizes glioblastoma is the amplification of the gene coding for *PDGFR*. This receptor is a transmembrane tyrosine-kinase receptor. Once activated by its ligand, it triggers an enzymatic downstream pathway trough MAP kinase cascade, and leads to cell proliferation, survival and migration. An overexpression of the PDGF receptor increases this phenomenon (Carrasco-Garcia et al., 2018; Song et al., 2018).

3.2.3. Vascular Endothelial Growth Factor (VEGF)

One of the most important molecular features in GBM is the overexpression of VEGF and its receptor (VEGFR). It correlates with the histological characterization of glioblastoma corresponding to an important vascularization, which is induced by VEGF. This blood vessel remodeling is also responsible for a reduction of infiltrative immune cells, protecting the tumor against a possible inflammatory response. As the vascularization allows tumor nutrition, VEGF is also involved in tumor growth (Turkowski et al., 2018).
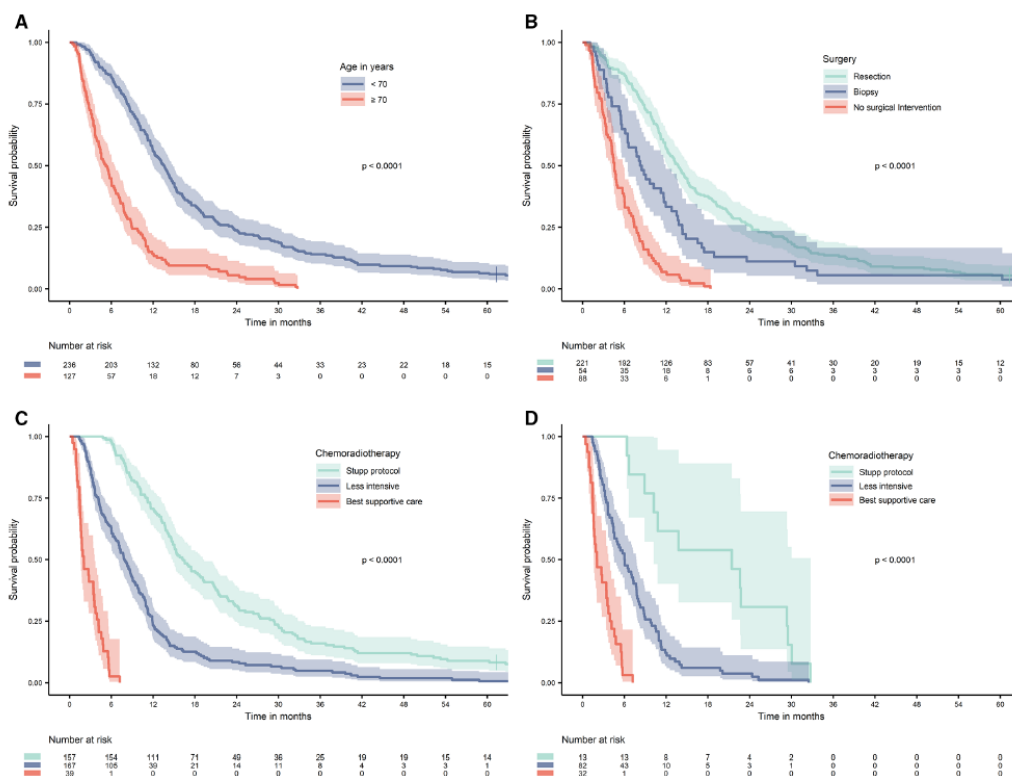
Figure 4. Measure of the Overall Survival (*OS*) in 363 adults diagnosed with glioblastoma between January 2007 and December 2014. (A) OS depending on age, median age at 70 years. (B) OS depending on surgery. (C) OS depending on chemotherapy treatment. (D) OS depending on radiotherapy with adjuvant chemotherapy. Surgery, radiation therapy and Temozolomide (*TMZ*) chemotherapy are the standard core for treatment (Bjorland et al., 2021).

3.2.4. Tumor protein 53

TP53 is a suppressor tumor gene coding for the p53 protein. This protein plays a fundamental role in many cellular mechanisms as cell cycle arrest, apoptosis, DNA repair, etc. As these processes aim to protect the cell from cellular damages, p53 is crucial for regulating cell proliferation (Noor et al., 2021). P53 protein is the protein which is the most frequently mutated in cancers in general. When mutated, it induces conformational changes, and the protein accumulates in the nucleus without exercising its role. It allows a permanent activation of the cell cycle thus a permanent proliferation state without any reparation of DNA damages (Sabapathy & Lane, 2019; Noor et al., 2021).

3.3. Epidemiology

Glioblastoma is the most malignant tumor of the CNS and which recurrence is inevitable. It represents about 54% of gliomas and 16% of primary tumors. The difficulty with glioblastoma at first, is to diagnose it. Indeed, clinical signs are for example: fatigue, headache, seizures or still anxiety. Those are common to benign conditions and represent such a real challenge. For this reason, most of patient were diagnosed after a hospitalization (McKinnon et al., 2021).

From the diagnosis, if the patient remains untreated, survival could be of 3 months. Standard of care therapy (see below) allows to increase Overall Survival (*OS*) until about 15 months. Only few patients survive more than 2 years (Witthayanuwat et al., 2018). In the USA, 3 per 100,000 glioblastoma cases are detected a year. The mean age at diagnosis is about 65-70 years. Above that age, survival decreases (**Figure 4**). Also, GBM incidence is 1.6 time higher in men than women. As for many cancers, there are genetic and environmental factors that may be pointed as causing factors: exposure to radiation, immune factors, or single nucleotide polymorphisms (Faleh Tamini et al., 2017; McKinnon et al., 2021).

3.4. Diagnosis

Glioblastoma suspicion is often detected by magnetic resonance imaging. Often, when identified, the tumor is already at an advanced stage. Before 2016, tumor diagnosis was based only on histological analysis of surgically resected tissue. Today, with the demonstrated importance of molecular features into the tumor classification, we talk about integrated diagnosis. After tumor resection, it is possible to analyze tumor genetic profile by genome or methylome profiling, different immunostainings, light or electron microscopy etc. Thanks to these methods, a combination of both histological and molecular features leads to an integrated diagnosis that also includes other key clinical information, in order to diagnose and assess the tumor type as precisely as possible (Alexander & Cloughesy, 2017; Louis et al., 2021).

3.5. Therapy

Treating glioblastoma is a real challenge due principally to its inter- and intratumoral heterogeneity (Alexander & Cloughesy, 2017). Many signaling pathways are perturbed and can help the tumor to escape therapy. Additionally, an immunosuppressive Tumor Microenvironment (*TME*) is also preventing therapy to efficiently kill all tumor cells. Drug-based therapies are complicated to develop because of the Blood-Brain Barrier (*BBB*) that prevents the therapeutic drug to reach the brain. By all these mechanisms, GBM is not sensitive to many therapies and is able to develop resistance (Ou et al., 2021). As recurrence is observed in almost all GBM cases, therapy so far only serves to prolong survival of several months, but do not improve quality of life (Fierro et al., 2022). Today, even if there is no efficient treatment against this brain tumor, there is a gold standard therapy that has been proven to be the most efficient to increase survival. Other therapies are studied to combine with the gold standard therapy or as monotherapy in order to prevent GBM recurrence.

3.5.1. Gold standard therapy for GBM

The standard-of-care for glioblastoma is a combination of three methods and is classically named the Stupp's protocol. After diagnosis, depending on the age of the patient, the tumor is safely maximally resected by surgery. When removed, the resected tumor tissue is used for integrated diagnosis. After surgery, patients undergo radiation therapy combined with an adjuvant that is temozolomide (*TMZ*), an oral alkylating agent. Indeed, it is considered that some tumoral cells have undoubtly escape the surgery as GBM are invading tumors. TMZ creates DNA damages and thus kills the tumor cells. This therapy is particularly efficient in patient carrying MGMT promoter hypermethylation, that prevents the expression of MGMT enzyme and therefore DNA repair mechanisms. On the other hand, patients that do not have their MGMT promoter methylated are less sensitive to TMZ chemotherapy. For example, rGBM has a variation in MGMT methylation and becomes resistant to this adjuvant therapy. As this standard-of-care is not sufficient to treat patients, research is still ongoing to completely eradicate GBM tumors, potentially by harnessing other GBM features. Most of the markers and genetic alterations that have been identified are studied, to find potential drug therapeutic targets (Tan et al., 2020; de Leo et al., 2021).

3.5.2. Targeted therapies

Targeted therapies consist in focusing on a special protein or gene that is mutated, downexpressed or overexpressed in glioblastoma. Against such molecules, it is possible to use monoclonal antibodies (*Ab*), small molecule inhibitors, or Antibody-Drug Conjugates (*ADC*) to block the overactivation or altered function of the protein, thereby decreasing tumor growth (E. K. Liu et al., 2020). In 2009, Nghiemphu and her team tested an antibody called *Bevacizumab* for glioblastoma treatment. This antibody is directed specifically against VEGF that is overexpressed in many cancers including GBM. The Ab anti-VEGF *bevacizumab* aims to prevent the binding of the growth factor on its receptor thus inhibiting the angiogenesis signaling pathway leading to a lack of tumor cell feeding, thus cell death and consequently, the decrease of tumor size (Nghiemphu et al., 2009).

3.5.3. Immunotherapies

This kind of therapy consists in helping patients' immune system to fight against tumor cells and showed a high efficacy for so-called "hot" tumors that are highly infiltrated by immune cells. A famous strategy is to block immune checkpoints (S. Xu et al., 2020). Immune checkpoints are molecules that regulate the immune response, by inducing a co-stimulatory or co-inhibitory signal after the binding to their receptor. These checkpoints are often expressed in tumors, defending tumor against immune system (Y. Zhang et al., 2020). The blockade of an immune checkpoint leads to the constitutive activation of immune cells without negative regulation, allowing a tumor specific response. PD1-PDL1 or CTLA4 are the most known and used targets. PD-L1 and CTLA4 are both part of the B7 immune checkpoint family. Both of them are involved in tumor progression by binding their receptor on T lymphocytes, which inactivate them leading to the decrease of an anti-tumoral immune response (Guan et al., 2021; Ni & Dong, 2017; Fierro et al., 2022). These checkpoint proteins are present in many cancers and are therefore good targets for immunotherapy. In melanoma, a treatment using *nivolumab* (anti-PD1) is FDA (*Food and Drug Administration*) approved, either alone or in combination with other drugs as *ipilimumab* (anti-CTLA4) (Koppolu & Rekha Vasigala, 2018). The efficiency of the anti-PD1 therapy for melanoma pushes to go further in the reflection and the hypothesis for the use of such a therapy in glioblastoma. Unfortunately, the blockage of PD1 in GBM was not efficient due to the low immune response and immunosuppressive TME of the brain tumor (X. Wang et al., 2019). However, the use of *pembrolizumab* (an anti-PD1) combined with *bevacizumab* seem to have a sustainable effect on recurrent GBM, which is encouraging for future therapies (Nayak et al., 2021).

A Dendritic Dell (*DC*) vaccine that increases antigen-presenting cells or the infection of tumor cells by an oncolytic virus thus releasing specific Tumor-Associated Antigen (*TAA*), use the immune response induction as potential therapy. It is also possible to induce the polarization of microglia into a form or another to direct the response to a pro-inflammatory response, preventing the establishment of an immunosuppressive micro-environment. It is called TAM therapy (de Leo et al., 2021). A last one, is the CAR T cell therapy. It consists in synthetic T Cell Receptor (*TCR*) on which was added a variable fragment (*Fv*) of immunoglobulin that recognizes specific tumor antigen.

4. Tumor microenvironment

Tumor Microenvironment (*TME*) is the environment that surrounds the tumor core into the brain. It contains nutrients, low pH, limited oxygen and an accumulation of metabolites that allow tumor growth. TME presents a high heterogeneity in its components. It is probably one of the features that lead to immunotherapy resistance. It is composed of different cell, such as macrophages, DCs, myeloid derived suppressor cells, neutrophils and microglia among others. Myeloid cells are the most present immune cell population in glioblastoma TME. Indeed, in a "cold" tumor such as GBM, there is very limited infiltration of immune T cells, which relates to the poor prognosis (de Leo et al., 2021). Myeloid cells are characterized by a high plasticity and include tumor-associated macrophages (*TAMs*) or glioma-associated macrophages in this case *(GAM)*, and microglia. Two types of TAMs co-exist. Microglia-derived Tumor Associated Macrophages (*MG-TAMs*) and Bone-Marrow derived Tumor Associated Macrophages (*BM-TAMs*) (**Figure 5**). Concerning microglia, they are Antigen-Presenting Cells (*APC*) of the brain. In GBM, microglial cells are mostly located in the peritumoral space. It has different roles in the brain as sensing all signals, allowing cell growth, regulating astrocyte function, or ensuring a defense function.

Both peripheral macrophages and microglia can differentiate into type 1 or type 2 macrophages/microglia upon reception of different signals (Geribaldi-Doldán et al., 2021). Microglia 1 (*M1*) induces a pro-inflammatory response, activating T lymphocytes from blood, either CD4$^+$ and CD8$^+$ directed against tumor cells, and TAMs1 that secretes pro-inflammatory cytokines in the perivascular space (Fu et al., 2020). Microglia cells can also polarize into type 2 (*M2*) leading to the release of anti-inflammatory cytokines activating BM-TAMs into type 2 TAMs (Yekula et al., 2020; de Leo et al., 2021), which lead to the decrease of cytotoxic T lymphocytes (Tumor Infiltrating Lymphocytes (*TIL*)) and of Natural Killer (*NK*) receptors activation. These processes are due to the constant interaction of glioblastoma cells with its tumor microenvironment to polarize T cells into T regulators cells (*Treg*) that are contributing to an immunosuppressive environment and releasing cytokines that permit tumor growth (Daubon et al., 2020).
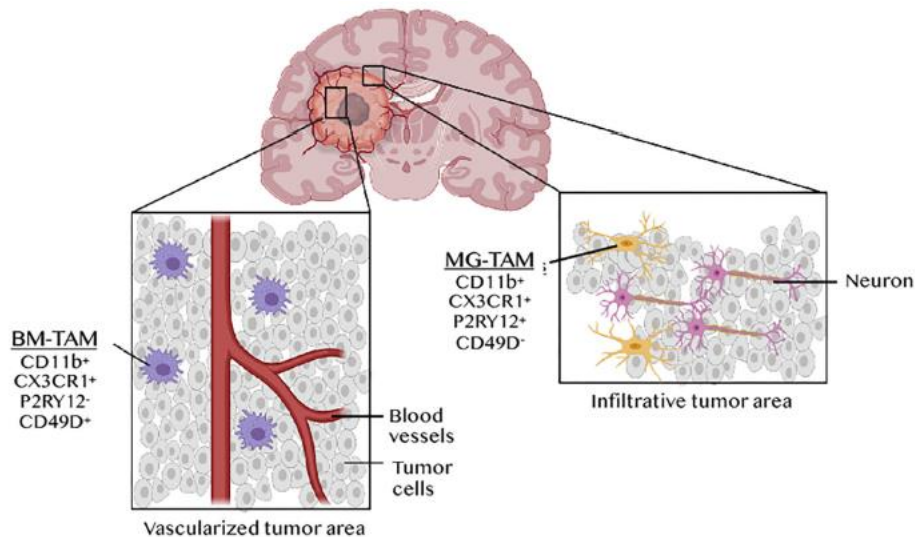
Figure 5. TAM in GBM. Tumor-associated macrophages (TAMs) arise from microglia (MG-TAMs) that are resident APC (Antigen-presenting cells) in the tumor core or from the bone marrow (BM-TAMs). These are monocytes differentiated into macrophages. They are smaller than MG-TAMs and able to infiltrate tumor mass compared to MG-TAMs that are found in the peritumoral area. They can be distinguished by the expression of different markers that are represented here (Daubon et al., 2020).

4.1.   T lymphocytes

In this research, we focused on T cells in glioblastoma. T cells are representing about 1% of immune cell population in the brain (Daubon et al., 2020). To be activated, they recognize antigens presented by APC via their TCR. As a reminder, APC present in the brain are microglial cells and BM-derived macrophages. The expression of different factors allows the activation of lymphocytes. TCR recognizes antigen presented on microglia cell surface leading to the differentiation and proliferation of activated T cells. To be fully activated, T cells need costimulatory signals thanks to molecules that are present on the T cell surface, binding receptors on APC (Janakiram et al., 2017).

T lymphocytes express on their surface CD3, which is a transmembrane protein associated with intracellular TCR chains, and able to activate T cell signaling cascade (Dong et al., 2019). This pathway contributes to the expression of different genes, such as CD69 which is an early activation marker leading to the production of certain cytokines as IL2 that acts in an autocrine way on T cells. A co-stimulator that is also necessary to activate completely these cascades, is the CD28 that binds B7 family receptors on other cells (Janakiram et al., 2017). It allows to increase the secretion of IL2 and thus induces the expression of IL2 receptor, CD25. This receptor present on T cell surface is a late activation marker (Y. H. Lee et al., 2017).

5. B7-H3

B7-H3, encoded by the *CD276* gene, is a transmembrane protein that is part of the B7 transmembrane family of proteins, also including PDL-1 (G. Chen et al., 2020). It has been shown to be overexpressed in many different cancers, such as glioblastoma. The B7-H3 mRNA is present widely in the different organs but the expression of the protein is rarely seen in healthy tissue, meaning that something happens at post transcriptional level. Indeed, during splicing, several elements intervene regulating the expression of the protein. The presence of micro-RNA 29 (*miR29*) is responsible of different B7-H3 expression profile and a duplication in exons can lead to different isoforms of the protein (L. Wang et al., 2014). These isoforms are made of an intracellular and an extracellular domain. The extracellular domain of 2Ig B7-H3 is composed of a variable and a constant immunoglobulin domain whereas the 4Ig B7-H3 isoforms is made of two variable and two constant domains. The intracellular tail is short and does not present any known active motif (Picarda et al., 2016) (**Figure 6**).

B7-H3 also exists into a soluble form. It is possibly cleaved from the surface by a metalloprotease and released in the medium or due to an alternative splicing in order to be secreted outside the cell (Fang et al., 2022).
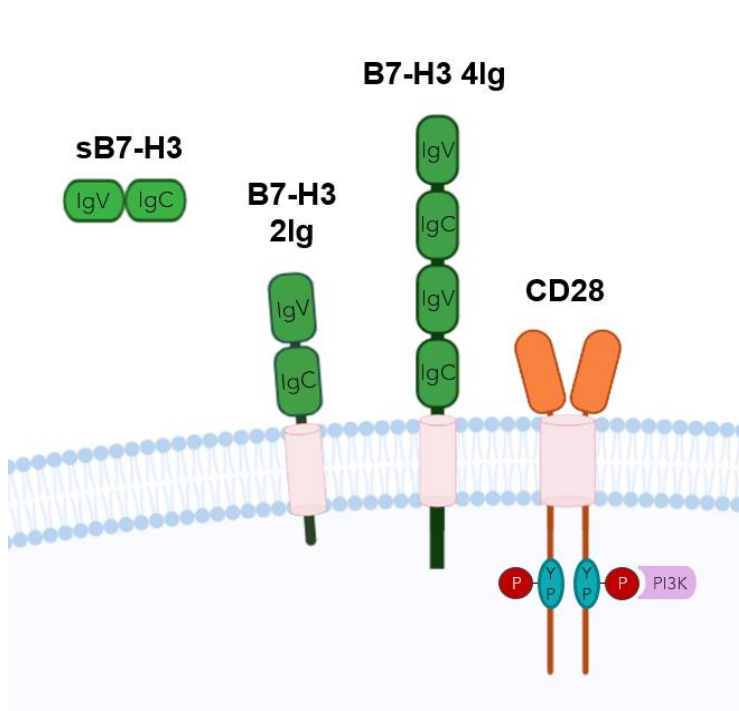
Figure 6. Representation of B7-H3 isoforms. B7-H3 2Ig is formed of IgC and IgV domain and is present in healthy tissue or rGBM. G. Zhang also described it as a basis for sB7-H3 after cleavage by a metalloprotease (G. Zhang et al., 2008). Also, this sB7-H3 is identified as an entire isoform, secreted by cell and is also composed as the 2Ig form. The 4Ig form is composed of a repetition of IgC and IgV domains and is present in GBM.
To demonstrate the shortness of B7-H3 intracellular tail, CD28 is figured. Its longer tail contains intracellular tyrosine motifs allowing the activation of PI3K signaling pathway. As a member of B7 receptor family, B7-H3 receptor might have the same composition.

As a member of the B7 transmembrane protein, B7-H3 acts as a co-stimulatory or co-inhibitory signal on immune cell regulation, however its role remains very controversial. Indeed, in normal tissue, B7-H3 inhibits Natural Killer (*NK*) cells activation and induces the release of pro-inflammatory cytokines by monocytes and macrophages. In adaptative immunity, it regulates T cell activation and its expression increases depending on the activation of T cells (Kontos et al., 2021). A non-immunologic role of this transmembrane protein is that it enhances the JAK2/STAT3 pathway that is regulating cell cycle by inhibiting apoptosis (H. Zhang et al., 2020), and inducing survival, proliferation of osteoblasts in physiological conditions or tumor cells. This controversial role of B7-H3 might be due to its both immune and non-immune related pathways. Indeed, by binding different receptors it may lead to the activation of different signals. In the same way, an additional challenge is to identify its receptor(s) to further investigate its role in physio pathological conditions.

In different cancers, B7-H3 is involved in an anti-tumoral response and acts also as an inhibitory immune checkpoint to promote tumorigenesis in others. To activate the T cell proliferation, it binds a receptor called Triggering Receptor Expressed on Myeloid (*TREM2*) Cell-Like Transcript 2 (*TLT2*) on mice T CD8^{+} cells to induce an adaptative response against tumor cells. Such a receptor has not been identified in human (Hashiguchi et al., 2008; L. Wang et al., 2014). B7-H3 seems also able to inhibit the adaptative immune response. B7-H3 is either localized on APC and tumor cells (Picarda et al., 2016), and is thus binding T CD4^{+} and T CD8^{+} to inhibit in the transcription factor NFκB (**Figure 7**). Without an effective transcription factor, proinflammatory cytokines are not produced and released in the extracellular medium (Kontos et al., 2021; Fang et al., 2022). Due to all these mechanisms, this checkpoint is probably responsible for drug therapy resistance. In solid cancers, B7-H3 is often correlated to a poor prognosis and is thus considered as a good therapeutic target.
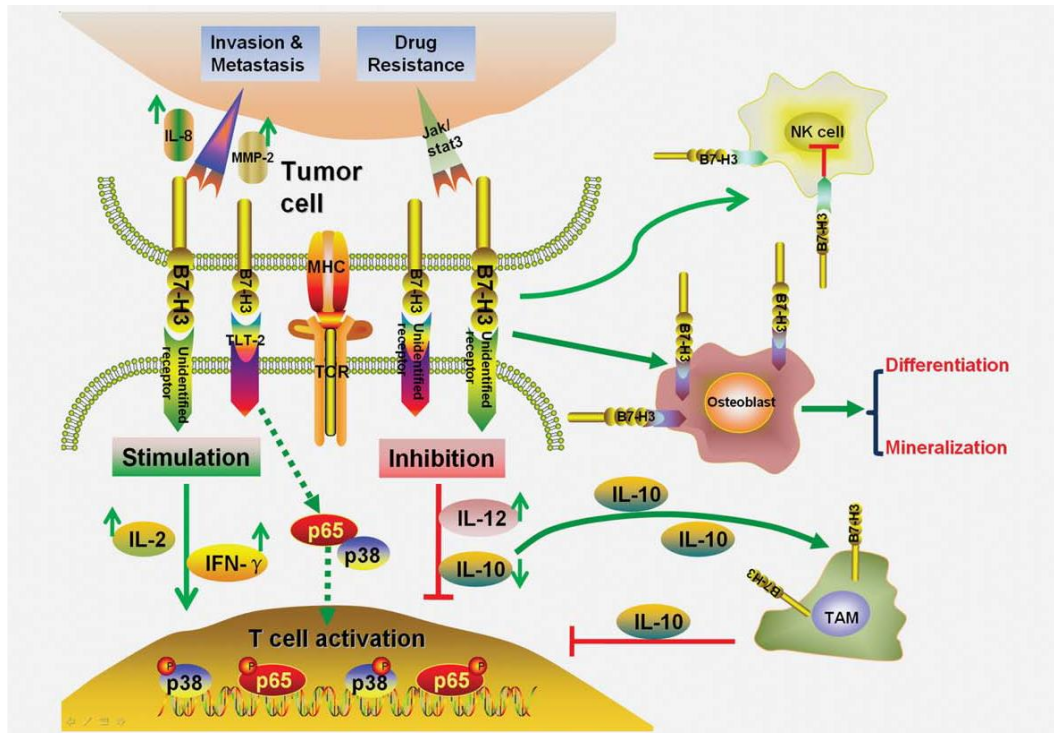
Figure 7. Representation of possible B7-H3 role(s) on tumor immune cell regulation. Depending on the receptor, B7-H3 is able to activate different pathways and transcription factors such as p65 and p38, leading to the secretion of different cytokines that are responsible for the activation (IL2, IFNγ, IL12) or inhibition (IL10) of the immune response acting on TAM, NK and T cells. It has a positive role on osteoblasts allowing their differentiation and formation of bones via mineralization. (L. Wang et al., 2014)

5.1. Therapeutic approaches targeting B7-H3

As B7-H3 plays different roles in tumors and has been identified to prevent tumor specific immune adaptative response, it might be a good target for therapies. Different types of therapies are possible, included immunotherapies. Targeted therapies are also possible as small molecule inhibitor, consisting in a drug directed against the protein of interest but as GBM is highly heterogenous, it is necessary to develop multitarget inhibitory molecules and is thus not a front-line choice for B7-H3 therapy (E. K. Liu et al., 2020).

5.1.1. Monoclonal Antibody (mAb) therapy

Monoclonal antibodies constitute a promising approach against immune checkpoints, such as B7 family members. Monoclonal antibodies are normally able to bind the immune checkpoint receptor to block the binding site and prevent the immune ligand on APC or tumor cells to link their receptor on T cells. B7-H3 receptor is not known yet, so the only mAb that was developed so far is directed against B7-H3 itself. It is a murine IgG1 and is called [131]I-*Omburtamab* or 8H9. However, it was shown to react with human solid tumors, such as gliomas. Its single chain Fv is targeting B7-H3 and seems really promising for radioimmunotherapy when coupled to [131]I (Ahmed et al., 2015). The radioisotope Iodine 131 emits two types of rays: β- and γ-rays. Coupled to the mAb directed against the protein of interest, the radioactive substance will be able to penetrate the tissue where the target is expressed and emits these two energies rays. β-rays will damage tumor cells expressing the protein while γ-rays serve as a diagnostic tool, to verify the targeting of the radioactive substance (Wyszomirska, 2012). [131]I-*Omburtamab*, directed against B7-H3, might then be a promising therapy for tumors expressing highly B7-H3 (Ménager et al., 2016; Yerrabelli et al., 2021).

5.1.2. Bispecific Antibody (bsAb)

Another immunotherapy approach consists into using bispecific antibody. Here, the monoclonal antibody against B7-H3 is coupled to a costimulatory protein, such as 4-1BB, also called CD137. This protein is found on T lymphocyte surface and is a member of the Tumor Necrosis Factor (*TNF*) superfamily. It has a costimulatory activity by binding its ligand on APC and inducing T cell proliferation, IL2 secretion, survival and cytolytic activity (Vinay & Kwon, 2014). The bsAb is thus targeting human B7-H3 on tumor cells, and mouse or human 4-1BB. B7-H3x4-1BB bsAb targets first lymphocytes in or around the tumor, forcing the specificity against B7-H3 and enhancing consequently T cell proliferation and cytokine production. After different tests, this therapy seems to be effective and safe either as monotherapy or combined with other target therapies (You et al., 2021).

5.1.3. Chimeric Antigen Receptor T cells (CAR T cells)

The last therapy that has already been developed is the CAR T cell therapy, which is based on the modification of T cells to specifically recognize a given antigen. These T cells express a chimeric antigen receptor (*CAR*), composed of (1) a TCR modified with an Ig Fv to recognize specifically the target protein, (2) the CD3 intracellular zeta chain that interacts with TCR to activate intrinsic activation pathways, and (3) costimulatory protein as OX40 and CD28 that bind their ligand or APC to amplify the activation signal (S. Xu et al., 2020). It is here possible to modify the TCR receptor thanks to lentivirus transduction, to recognize B7-H3. The challenge in this therapy lies in the expression level of the protein in tumor cells. B7-H3 expression is heterogeneous among a tumor. B7-H3 CAR T cell will focus on cells that express B7-H3 at a certain level, allowing to target specifically tumor cells, but will not target cells expressing a lower level of B7-H3, which may then escape treatment and take part of the recurrence (Picarda et al., 2016; Nehama et al., 2019; Tang et al., 2019).

6. B7-H3 in glioblastoma

B7-H3 protein is detected in most gliomas, and its expression level is correlated with malignancy (Tang et al., 2019). That explain its higher expression in grade 4 glioblastoma. Out of the two isoforms of the protein that exist, 4Ig B7-H3 is specific for brain tumor and especially glioblastoma and the 2Ig B7-H3 isoform is found in normal, healthy brain. However, this 2Ig B7-H3 isoform is overexpressed in GBM recurrence (Digregorio et al., 2021). Although the roles of B7-H3 have been identified and are more and more elucidated in hematologic or other tumors, it is not clear in glioblastoma whether B7-H3 acts as a costimulatory or coinhibitory immune checkpoint, or if its role is only non-immunologic, acting on tumor growth via intrinsic signaling cascades. For example, B7-H3 has recently been identified to play role in angiogenesis, responsible for the expression of VEGF, increasing the malignancy and tumor aggressiveness (Folkins et al., 2009).

Objectives of the work

Glioblastoma is the most common and aggressive primary brain tumor in the central nervous system. It is characterized by a certain heterogeneity, either intra- or intertumoral. The objective of this research is to determine the role of B7-H3 protein, that has been shown to be overexpressed in many different cancers and seems to play a role in tumor progression. This transmembrane protein is present in GBM, but its role in tumor growth and recurrence is very controversial. In this work, we first aim to identify its "tumor-intrinsic" role, to understand the role of B7-H3 inside the cell and whether it acts on the regulation of tumor-related processes (thanks to different cell lines). Given that B7-H3 is part of the B7 super family and considered as an immune checkpoint, we also aim to verify its action as a costimulatory or coinhibitory signal. We will analyze the "tumor-extrinsic" role of B7-H3 by analyzing its possible effect on T cells from the TME. This is done here by coculturing GBM cells (expressing variable levels of B7-H3) with T cells and observing the effects of the protein on immune cell activation via flow cytometry.

Material and methods

1. Glioblastoma cell lines and culture

We used three different human GBM U87MG (named hereby U87) cell lines. Two cell lines were transduced with CRISPR-Cas9 system. A part of U87 cells were transduced with a guide-RNA Non-Target cocktail (the second cocktail was the most appropriate), called **U87 gRNA NT₂** *(Non-Target cocktail 2)*. The other cell line was transduced with a guide-RNA directed against the gene coding for human B7-H3 protein and is called **U87 gRNA hCD276 KO** *(Knock-out)*. The last U87 cell line is called **U87 shNT OE hCD276 2Ig** *(Overexpressed)*, where B7-H3 has first been overexpressed to regenerate its expression, and serves as a positive control. We cultured these cells in adherence in a 2D culture medium which is complete DMEM composed of DMEM High Glucose (#L0104-500, Biowest, France), with 10% FBS (*Fetal bovine Serum*), 1% Penicillin-Streptomycin Solution 100x (#L0022-100, Biowest).

We used also various patient-derived glioblastoma stem like cells T08, T013, T018 and T033. These are cultured as neurosphere in a 3D culture medium called NPC medium, made of DMEM F12 supplemented by B27 without vitamin A, Penicillin-Streptomycin, Normocin and Heparin.

2. T-lymphocytes coculture

Before cultures, we need to extract lymphocytes from donors' blood. After collecting blood, we did different samples by filling 15 mL falcon tube with 4.5 mL of blood. To extract T cells from these samples, we add to each one 225 µL of RosetteSep™ HLA Lymphoid Cell Enrichment Cocktail (#15271HLA, StemCell Technologies, Canada). We incubated samples 20 minutes under agitation before dilute them twice in RPMI 1640 Medium with L-Glutamine (Lonza, #12-702Q) and homogenize.

In a SepMate™ - 15 (IVD) (#85415_C, StemCell Technologies) tube, we added 6 mL of RosetteSep™ DM-L Density Medium (#15705, StemCell Technologies) and gently poured the blood on top to prevent the mix of both. We centrifuged the samples at 1,258g for 10 minutes.

We then recovered the supernatant and centrifuged 5 minutes at 700g. Lymphoid cells are forming a small red cap, that we can isolate and further use for co-culture with GBM cells. We recovered 100,000 lymphocytes, activated them with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (#10970_C, StemCell Technologies) and put them in presence of 100,000 U87 cells from each cell line, in a medium ImmunoCult™-XF T Cell Expansion Medium (#10981, StemCell Technologies) before analyzing lymphocyte activation by flow cytometry (see 4. Flow cytometry).

3.   Animals and stereotactic injection

For *in vivo* experiments, we injected U87 cell lines or patient-derived glioblastoma stem like cells (*GSCs*) in Swiss Nude mice (Crl:NU(lco)-Foxn1nu). These mice are athymic, and thus devoid of any adaptative immune system. In normal mice, development of a tumor after injection of human glioblastoma cells would be limited, since the mouse immune system would recognize and kill human tumor cells.

We injected directly in mice brains using a stereotactic frame. The mouse was thus placed in a stereotactic framework and anesthetized with a v/v (*volume/volume*) mixture of xylazine and ketamine. To inject, we used Bregma (the junction point of the sagittal and coronal sutures) as a "0" landmark. From Bregma, the injection syringe is moved of 2 mm on the right, 2.5 mm straight and 0.5 mm deep from the surface of the brain. We slowly injected 50,000 U87 cells or 100,000 GSCs in 2μL of PBS and removed the syringe gently to prevent the cell aspiration.

At tumor endpoint, after 3 or 4 weeks approximatively, we sacrificed mice to recover the brains. We first put them to sleep with a solution of Euthasol. We perfused them intracardially with saline solution to rinse the tissue from blood then with PFA 4% to fix the tissue. Once these steps done, we cut the skull and recovered the brain. The following steps aim to conserve the brains until their use for other experiments. They were fixed in PFA 4% for 24H then conserved in sucrose [sucrose 30% and azide pH7.4] before being embedded in OCT for cryoconservation. To use them for immunostainings, we cut them in 14 μM thick sections using a cryostat.

Antibody	Species	Dilution	Reference
Anti-CD276	Polyclonal Rabbit	1/250	ab226256 (Abcam, UK)
Anti-Iba1	Polyclonal Goat	1/1000	ab5076 (Abcam)
Anti-hVimentin	Monoclonal Mouse	1/200	MAB3400 (Merck, Germany)
Anti-Nkp46	Polyclonal Rabbit	1/100 or 1/500	PA5-102860 (Thermofisher, USA)

Table 1. Primary antibodies

Antibody	Species	Dilution	Fluorochrome	Reference
Ab anti-rabbit	Donkey	1/500	Far red	
Ab anti-rabbit	Donkey	1/500	Red	
Ab anti-rabbit	Donkey	1/500	FITC	Jackson ImmunoResearch, UK
Ab anti-goat	Donkey	1/500	Far red	
Ab anti-mouse	Donkey	1/500	FITC	

Table 2. Secondary antibodies

4.   Immunostaining

4.1.   Immunofluorescence

Depending on the sample, the procedure changes a little. We used either adherent cells, sections of patient-derived GSC neurospheres or mice brains tissue. For cells, we first recovered them from culture by passing them. We removed the 2D medium complete DMEM, washed cells with PBS (*Phosphate-Buffered Saline)* (#BE17-512F, Lonza, Switzerland) and detached cells with 0.05% Trypsin-EDTA 1x (#25300-054, Gibco, USA). We retrieved cells, counted them and seeded them on glass coverslips. We later fixed them in PFA 4%, before conserving. Patient-derived GSC neurospheres were also fixed in PFA 4%, before being sectioned using the cryostat.

Once brain sections and neurospheres were on slides, we fixed them one more time with PFA 4%, then washed them with PBS and let it dry before putting them in the oven at 60°C for 30 minutes. We washed in 3 alcohol baths 50%, 70% and 100% for 5 minutes each. We then washed 2 times with water for 2 minutes. All these steps prevent tissue detachment from the glass slide. To make epitopes accessible for antibody binding, we performed antigen-retrieval with Tris-EDTA buffer pH9 [Tris base 10mM, EDTA 1mM, 0.05% Tween 20] in a pressure pot at 99°C, waiting 3 minutes from the moment the steam comes out and let it cool for about 30 minutes at RT. The next step is the permeabilization step, to detect proteins inside the cell, thanks to PBS-Triton 0.1% [Triton diluted in PBS] for 10 minutes, wash 2 times 5 minutes in PBS. To avoid tumor auto-fluorescence, we then treated slides with TrueBlack 1x [TrueBlack 20x diluted in 70% ethanol], for 30 seconds, and washed 3 times for 5 minutes with PBS.

For the coverslips, we started from the following step. We used a blocking solution [PBS with 10% NDS (*Normal Donkey Serum*)] that blocks non-specific sites as the secondary Ab is a donkey Ab and let the solution on the slide for 45 minutes. We also used NDS to dilute primary Ab [PBS with 1% NDS]. We incubated the sections with the primary Ab [**Table 1.**] ON (*OverNight*) at 4°C, or for 1 to 2 hours at RT (*Room Temperature*). The next day, we removed the primary antibody solution and washed the slides 3 times with PBS for 5 minutes, before incubating them with the secondary Ab solution [secondary Ab (**Table 3.**) diluted in PBS], for 1

until 2 hours at RT. Again, we washed 3 times with PBS for 5 minutes and DAPI for 5 minutes [1/10,000 in H_2O]. We washed then in water and let dry. Once completely dry, we mounted the slide using a permanent histological mounting medium. For detection, we used an epifluorescence microscope (Zeiss Apotome Axio Observer).

4.2. Immunohistochemistry

We did immunostaining on tissue sections. We used a Kit (Enzo Polyview® Plus HRP-DAB (anti-Mouse)) for detecting the tumor through the labelling of human vimentin. We put the tissue-mounted slides baking at 60°C for 30 minutes to fix the sections to the slide. In the second step, we aimed to unmask epitopes thanks to an Antigen Retrieval Solution [10 mL of Antigen Retrieval Reagent (10x) in 90 mL of deionized H_2O]. For retrieving antigen, we put the slides in a pressure-cooker for 3 minutes at 99°C and cooling at RT. We washed with PBS-T [wash buffer composed of 500 µL of Tween-20 in 1L of PBS]. This technique is based on an enzyme, here HRP. To allow tumor detection thanks to HRP labelling, we inactivated endogenous peroxidase activity by incubating sections in Peroxidase Block Solution 8 minutes at RT. We then washed this solution with PBS-T for 5 minutes. Next, we blocked non-specific sites to improve antigen fixation specificity. For that step, we used the Antibody Blocker/Diluent for 10 minutes, before washing twice slides in PBS-T for 5 minutes. We let then incubate ON at 4°C (or 2 hours at RT) the primary antibody Mouse anti-hVimentin (**Table 1.**). After a night, we washed 3 times with PBS-T for 5 minutes each. We repeated the Antibody Blocker/Diluent step for 10 minutes and washed twice for 5 minutes. We then added POLYVIEW® PLUS HRP Anti-Mouse Reagent and incubated for 1H at RT. We washed next slides 3 times with PBS-T for 5 minutes each. Under the hood, we added DAB Chromogen/Substrate [40 µL of HIGHDEF® DAB Chromogen in 1mL of HIGHDEF® DAB Substrate Buffer] for 5 minutes, still at RT and washed slides with water. We counterstained the slides with HIGHDEF® Hematoxylin for 1 or 2 minutes at RT and washed them in water. We let them dry before mounting by using a mounting medium. To quantify tumor volume, we used a quantification and histological cartography software *Mercator* (Explora Nova).

Antibody	Species	Dose (μL)	Fluorochrome	Reference
Anti-CD276	Monoclonal Mouse	5	APC	135608 (BioLegend®, USA)
Anti-hCD45	Monoclonal Mouse	5	V500	560777 (BD Horizon™)
Anti-CD3	Mouse	5	APC-H7	641415 (BD Horizon™)
Anti-hCD25	Monoclonal Mouse	5	RPE	R081101-2 (DAKO, Denmark)
Anti-hCD69	Monoclonal Mouse	5	PE-Cy™7	335792 (BD Horizon™)
Anti-hCD276	Monoclonal Mouse	5	BV421	565829 (BD Horizon™)
IgG1 κ isotype Control	Mouse	5	BV421	562438 (BD Horizon™)
Anti-hCD152	Monoclonal Mouse	20	APC	555855 (BD Horizon™)
IgG1 κ isotype control	Mouse	5	APC	340442 (BD Horizon™)
Anti-hCD56 (NCAM-1)	Monoclonal Mouse	5	BB515	564488 (BD Horizon™)
7AAD		5	PerCP	A07704 (Beckman Coulter, USA)

Table 3. Antibody for Flow cytometry

5. Flow cytometry

To recover cells, we dissociated them thanks to accutase, either for 2D and 3D cell cultures. We suspended cells in flow buffer [PBS supplemented by 1% of BSA (*Bovine Serum Albumin*), 1mM EDTA, and 0.1% Azide] in 100 µL in a flow cytometry tube with at least $1x10^5$ cells. We added antibody from **Table 2.**, mixed the sample and incubated for 20 minutes to 1H RT in the dark. We then had to wash cells by adding flow buffer in the tube and centrifuged 400g for 4 minutes at 4°C. In the following step, we removed the supernatant keeping the cap of cells and repeated the wash step. Next, we resuspended in flow buffer 500 µL and added 500 µL of fixation buffer [PFA 1%] and mixed. We let incubate the tubes for 15 minutes at RT. We washed again cells 2 times with PBS and centrifuged the suspension at 4°C for 4 minutes. Again, we removed supernatant and resuspended cells in 300 µL of flow buffer in flow tubes. Finally, these tubes were passed in the cytometer FACS Canto (BD FACSCanto™ II System). Data were analyzed with FlowJo™ Software.

For cells in coculture, principally lymphocytes, we recovered the supernatant and centrifuged once 5 minutes at 700g. We then eliminated the supernatant and washed once or twice with PBS, centrifuging between washes still 5 minutes at 700g. Again, we eliminated the supernatant and resuspended in 300 µL of PBS. In flow tube, we put 100 µL of the suspension and incubated 20 minutes in obscurity at RT with the antibodies directed against the proteins of interest. Next, we washed these tubes and fixed them in a BD FACS Lyse Wash Assistant (#337146 BD Horizon™, USA) before passing in the cytometer FACS Canto (BD FACSCanto™ II System). Finally, we analyzed with Kaluza Analysis Software.

6. ELISA test

We used of ELISA kit (R&D Human B7-H3 Quantikine ELISA Kit, #DB7H30) to quantify the concentration of soluble B7-H3 in the cell culture medium.

In a 96 wells plate, we put 500,000 cells/well, in 3 mL of complete medium. After 24H, cells should adhere to the well surface, we then discarded the medium and changed it to 1 mL of DMEM High Glucose (L0104-500, Biowest) without FBS and PS. We let cells incubate for

40H. After that period of time, we recovered the supernatant. We then filtered this supernatant on a 0.22 µM filter.

We reconstituted Human B7-H3 Standard with 1 mL of deionized water to obtain a concentration of 500 ng/mL. For the standard curve, we prepared serial dilutions. We collected 100 µL of the Standard Solution that we diluted in 900 µL of Calibrator Diluent RD6-41 to make a 50 ng/mL dilution. From that tube, we collected 500 µL and diluted it in Calibrator Diluent RD6-41. We repeated this operation to dilute 6 times the Standard 500 ng/mL. We had to let all the reagents at RT before beginning the experiment. We prepared the different required solutions before the beginning of the experiment. We placed as many strips as necessary in the frame and added 100 µL of Assay Diluent RD1-109 per well. We then added 50 µL of Standard, Control (Calibrator Diluent serves as control 0 ng/mL) or sample to each well. We mixed by taping gently the plate on the bench, covered with plate adhesive and let it incubate for 2H at RT on a microplate shaker. Then, we aspirated liquid in each well and wash 4 times with 400 µL of Wash Buffer [20 mL of Wash Buffer Concentrate in 480 mL of deionized water] per well.

In the next step, we added 200 µL of anti-Human B7-H3 Conjugate Antibody in each well and covered with a new adhesive before letting it incubate for 2H at RT. Again, we aspirated and washed 4 times with Wash Buffer. We then added 200 µL of Substrate Solution [volume-volume of Color Reagent A and B] in each well and let incubate 30 minutes at RT in the dark. Finally, in each well, we added 50 µL of Stop Solution and read the plate absorbance at 450 nm in the 30 following minutes.

7. EdU proliferation assay

We retrieved cells from culture by passing them. We thus removed the 2D medium complete DMEM, washed cells with PBS (#BE17-512F, Lonza) and detached them from their support with 0.05% Trypsin-EDTA 1x (#25300-054, Gibco). We inhibited trypsin Trypsin-EDTA by adding culture medium containing FBS. After centrifugation, we resuspended them in complete DMEM and counted them to put about 5,000 cells/well in a 96 wells plate. We then let incubate ON and changed medium to SS FBS medium [DMEM High Glucose (#L0104-500,

Biowest), 1% Penicillin Streptomycin (#L0022-100, Biowest), 0.2% Normocin], which is devoid of serum. Again, we let incubate ON to eliminate FBS from culture and synchronize cell cycle. Next, we changed medium with another one containing FBS, CM [DMEM High Glucose (#L0104-500, Biowest), 10% FBS, 1% Penicillin Streptomycin (#L0022-100, Biowest), 0.2% Normocin], to be sure cell restart proliferate.

We treated cells ON with AraC 2 µM and FUdR 10 µM, added in the culture medium to prevent disturbances (these molecules are chemotherapy agents that inhibit proliferation and serve as negative control for the experiment). At the same time, to prevent the premature inactivation of inhibitor, we treated the non-control wells with EdU. We added 10 µM into the culture medium and incubate for 3H. Then, we fixed cells in 4% PFA for 10 minutes at RT or 8% PFA v/v keeping the medium to minimize cell loss. After that, we aspirated medium containing 8% PFA and washed 2 times with PBS-T for 5 minutes and once with PBS also 5 minutes.

We prepared 50 µL/well of staining solution [Tris 100 mM, $CuSO_4$ 2mM, Alexa Fluor™ 488 Azide 2 µM, Ascorbic Acid 100 mM, dH_2O] and let incubate cells for 20 minutes at RT in the dark. We washed cells 3 times with PBS-T for 5 minutes. In the next step, we stained nucleus with DAPI [1:10 000 in PBS-T] for 5 minutes at RT. Again, we washed cells in PBS-T 2 times for 5 minutes and once in PBS for 5 minutes too. At last, we imaged cells and determined cell proliferation using detection of EdU positive cells (coupled to Alexa Fluor) compared to a total number of cells colored in DAPI.

8. Statistics

Statistical analysis was done via GraphPad Prism 8.0.1 Software. We first checked for the data normality to direct the right statistical analysis. Differences between two groups were then assessed using Student's t-test or ANOVA. The statistical significance level was fixed at p=0.05.
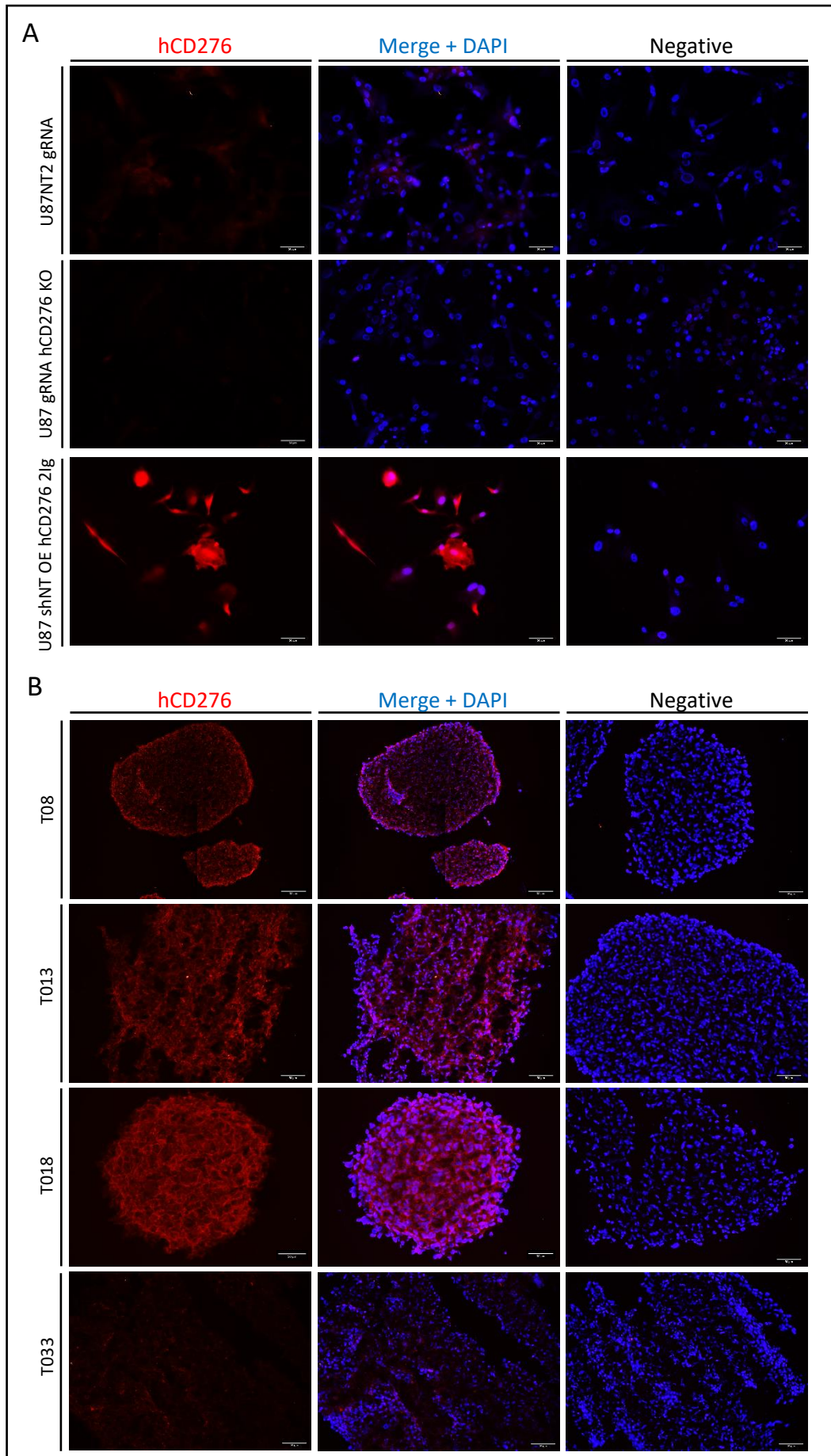
Figure 1. Immunofluorescence staining for CD276 detection in A. U87 cell lines NT2, KO and OE and in B. patients derived glioblastoma stem-like cells T08, T013, T018 and T033 with Ab anti-CD276 (**Table 1.** and **Table 2.**)

Results

1. B7-H3 expression in U87 cells and patient-derived GBM stem-like cells

To evaluate the expression of B7-H3 in patient-derived GSCs, we investigated different techniques as immunostainings and flow cytometry. We used the three U87 cell lines to validate our labeling using our B7-H3 antibody (**Table 1**.) and its adequate concentration in order to applicate it on patient-derived GSCs. In the U87 gRNA NT2, gRNA hCD276 KO and shNT hCD276 OE 2Ig cell lines, we see respectively a basal signal, no signal and a significantly more intense signal corresponding to the B7-H3 protein, as expected (**Fig. 1A**). We then applied those working experimental conditions to patient-derived GSCs T08, T013, T018 and T033. We observe a certain intensity of fluorescence that varies a little between the four tumors (**Fig. 1B**).
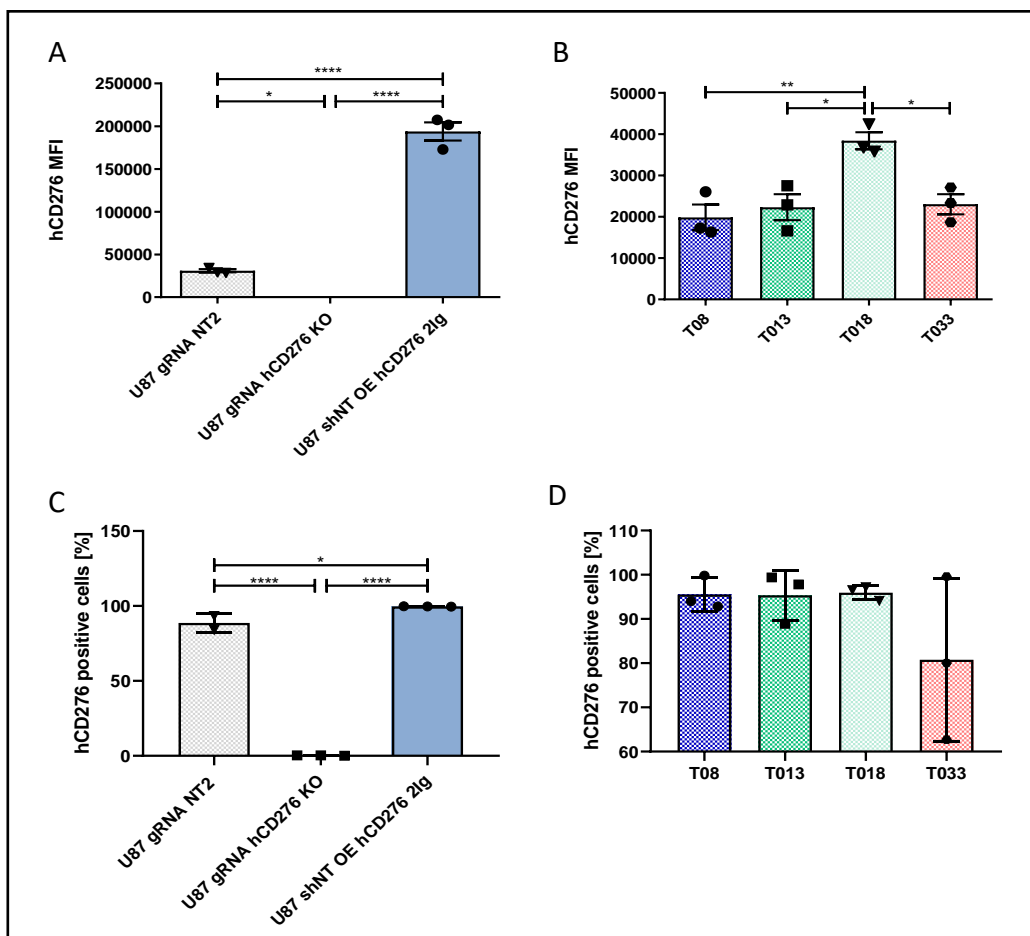

Figure 2. Flow cytometry of U87 cell lines and patient-derived GSCs with an anti-CD276 Ab coupled to APC fluorochrome (**Table 3.**) measuring Mean Fluorescence Intensity (*MFI*) in A. U87 cell lines *p*<0.0001 and in B. patient-derived GSCs *p*=0.0050. Percentage of cells positive for CD276 in C. U87 cell lines *p*<0.0001 and in D. patient-derived GSCs *p*=0.2439 ns.

To confirm these results in a quantitative manner, we used an antibody against B7-H3 coupled to APC (**Table 3.**) and performed flow cytometry analysis. We used U87 cell lines, analyzing their Mean Fluorescence Intensity (*MFI*) (**Fig. 2A**). We observe the same results as shown in the immunostaining, i.e., a low fluorescence intensity (30,905 ± 3,198 MFI units) for U87 gRNA NT2 cell line, no fluorescence for U87 gRNA hCD276 KO and a high intensity signal (193,901 ± 18,494 MFI units) for U87 shNT hCD276 OE 2Ig, as expected. When we look at the number of cells that are expressing the protein, we see that the number of positive cells is quite high (88.68 ± 6.61%) for U87 gRNA NT2, none for gRNA hCD276 KO and still high (99.67 ± 0.15%) for U87 shNT hCD276 OE 2Ig cells (**Fig. 2C**). After validation of the Ab, we evaluated the expression of the protein in patient-derived GSCs depending on MFI and on CD276 positive cells. Respectively, we see a significant difference in fluorescence intensity between T018 and others (**Fig. 2B**) and the number of positive CD276 cells is quite the same in the different lines (**Fig. 2D**).

2. Presence of soluble B7-H3 (sB7-H3)

As mentioned earlier, a soluble form of the B7-H3 exists. It has been investigated in the laboratory to assess the presence of this sB7-H3 in the medium of cell culture.
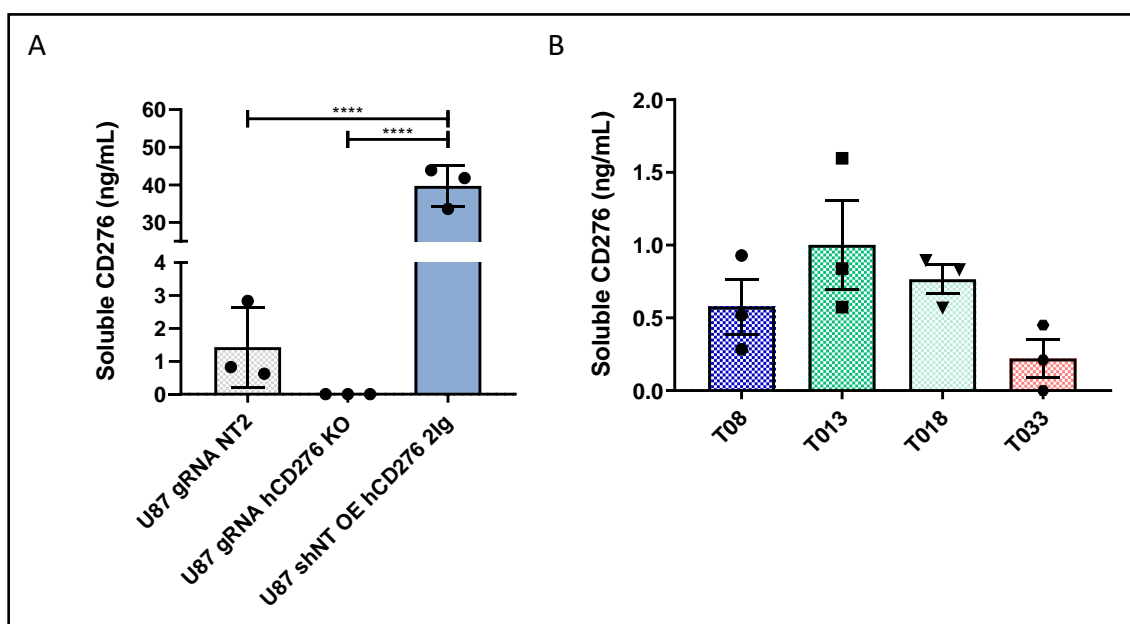

Figure 3. Detection of sCD276 in culture medium using A. U87 cell lines *p*<0.0001 before testing B. patient-derived GSCs *p*=0.1092 ns.

Again, U87 cell lines were used to validate the experiment. We observe a significantly higher amount of sB7-H3 (39.75 ± 5.42 ng/mL) in U87 shNT hCD276 OE 2Ig culture medium than in U87 gRNA NT2 (1.43 ± 1.22 ng/mL), whereas no sB7-H3 is detected in U87 gRNA hCD276 KO medium (**Fig. 3A**). The analysis of GSCs T08, T013, T018 and T033 culture medium showed the presence of sB7-H3 but a no significant difference between the different cultures (**Fig. 3B**).

3. Role of B7-H3 in cell proliferation

To investigate the role of B7-H3 in cell proliferation, collaborators in the laboratory performed an EdU assay consisting in measuring cell proliferation rate. This aims thus to test if there is a difference in tumor growth via testing *in vitro* tumor cell proliferation in presence and in absence of B7-H3. Consequently, U87 gRNA NT2 and U87 gRNA hCD276 KO cell lines are used for this test (**Fig. 4**). The graph does not show any significant difference in proliferation between the two cells lines, in presence or in absence of B7-H3.

Figure 4. Assessment of B7-H3 in tumor proliferation through EdU assay *p*=0.8499 ns.
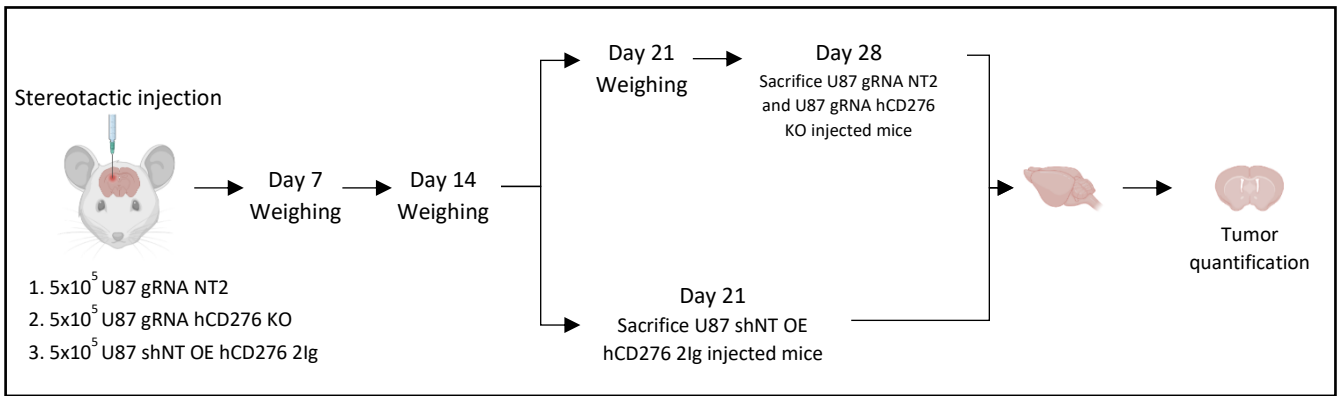
Figure 5. Representation of the injection of 5×10^5 of the three U87 cell lines U87 gRNA NT2, U87 gRNA hCD276 KO and U87 shNT OE hCD276 2Ig in mice brains. Mice were weighted each week and sacrificed at day 21 for OE injected mice and at day 28 for NT2 and KO injected mice.
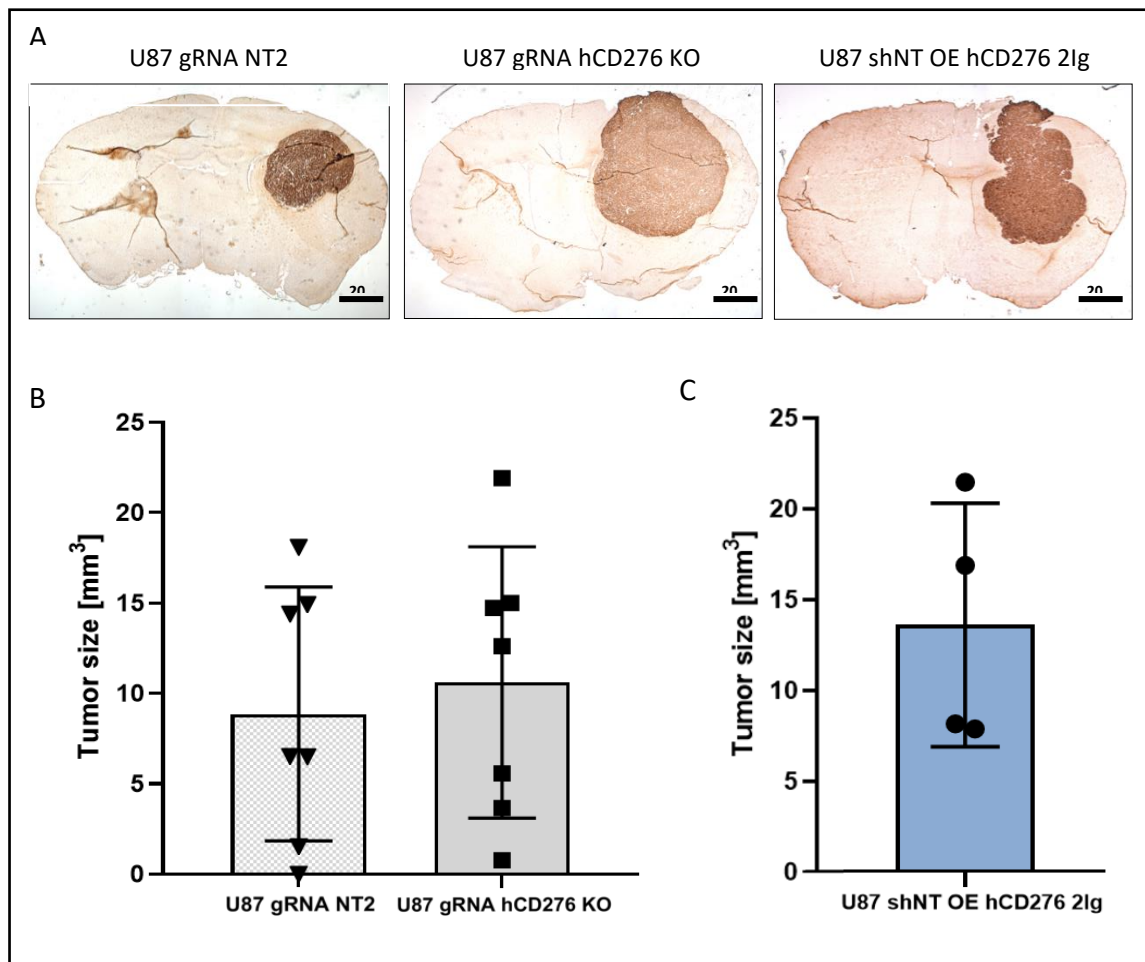
Figure 6. Results of the tumor quantification based on slices of brains injected with the different U87 cell lines U87 gRNA NT2, U87 gRNA hCD276 KO and U87 shNT hCD276 OE 2Ig. A. Brains slices with tumor colored through immunohistochemistry reaction with DAB (Material and Methods 4.2.) thanks to an anti-human Vimentin Ab (**Table 1**.) B. Measure of tumor size depending on NT2 and KO cell type injected in mice brains in mm^3 p=0.6605 ns. C. Measure of tumor size in U87 shNT hCD276 OE 2Ig mice.

4. Role of B7-H3 in tumor growth

The role of B7-H3 was tested *in vitro* in cell proliferation but we also investigated the role of B7-H3 in tumor growth *in vivo* after injection of different cell lines directly in mice brains. We injected the three U87 cell lines in nude mice brains (**Fig. 5**). At day 0, 5×10^{4} U87 gRNA NT2, U87 gRNA hCD276 KO an U87 shNT OE hCD276 2Ig cells were injected through stereotactic injection directly into the striatum. Mice were weighted at weekly intervals. Mice that received U87 gRNA NT2 and U87 gRNA hCD276 KO mice showed clinical signs of disease at day 28, considered as tumor endpoint, and were therefore sacrificed. U87 shNT hCD276 OE 2Ig mice were sacrificed at day 21 for experimental reasons that are independent of this work. On the brains sections (**Fig. 6A**), we do not observe any big difference in the tumor size. The U87 gRNA NT2 tumor size is nevertheless a little smaller than the U87 gRNA hCD276 KO tumor. Quantification of the tumor volume on serial sections of U87 gRNA NT2 (8.86 ± 2.66 mm^3) and U87 gRNA hCD276 KO (10.61 ± 7.51 mm^3) in **figure 6B** shows no significant difference, with a *p*>0.05. The tumor volume in mice injected with U87 shNT hCD276 OE 2Ig cells is not directly comparable (given the different sacrifice timing) but indicates that U87-OE cells are able to form tumors as well (**Fig. 6A and 6C**).
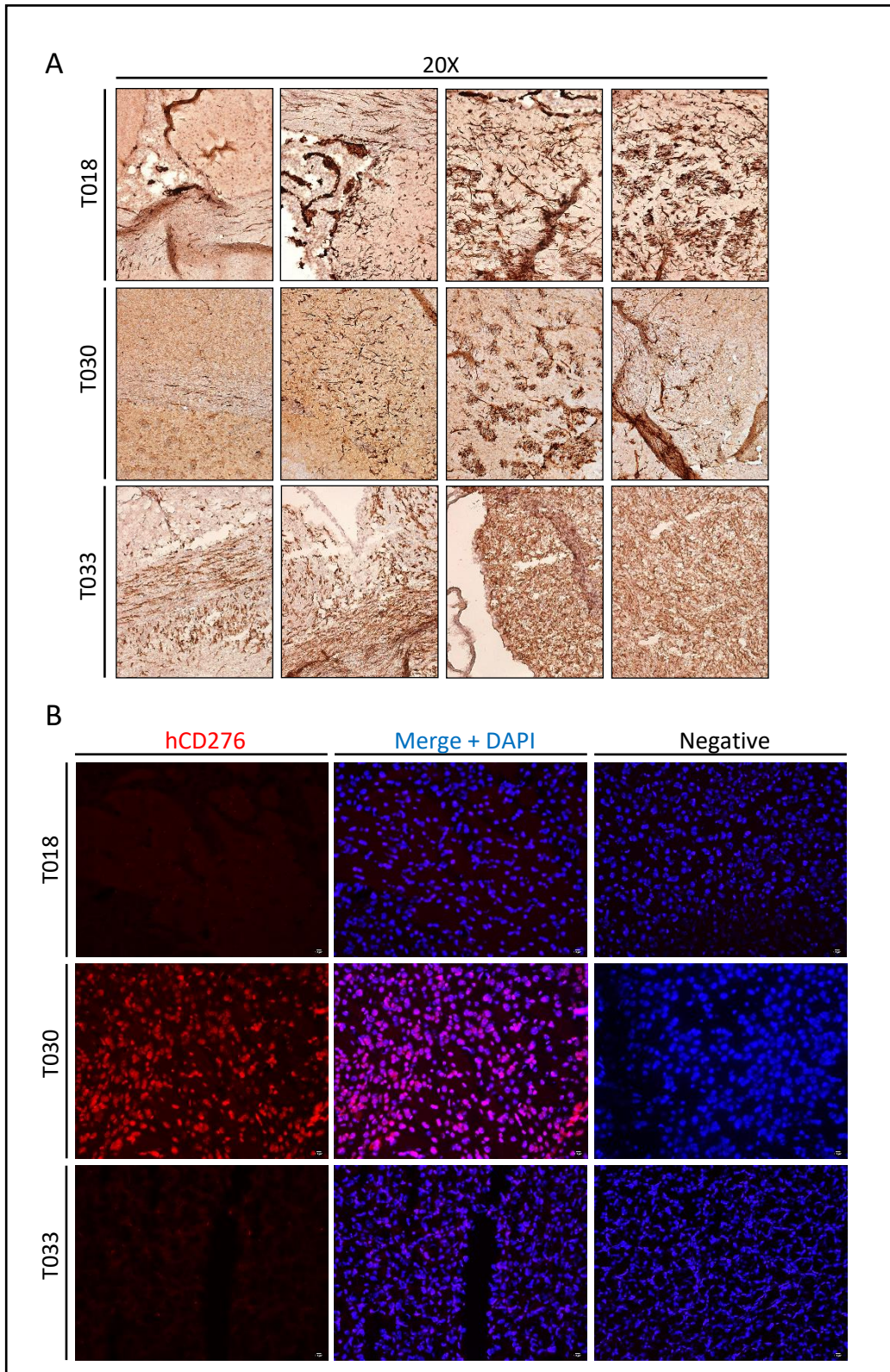
Figure 8. Immunostaining on mice brains injected with GSCs T018, T030, T033. A. Immunohistochemistry with DAB reaction (Material and Methods 4.2.) targeting tumor cells thanks to an Ab directed against human Vimentin (**Table 1**.). B. Immunofluorescence targeting B7-H3 in GSCs injected in mice brains (**Table 1**. and **Table 2**.)

5. Detection of B7-H3 in GSCs implanted *in vivo*

We aimed to detect B7-H3 *in vivo* via immunostaining after injection of the different cell lines in mice brains.
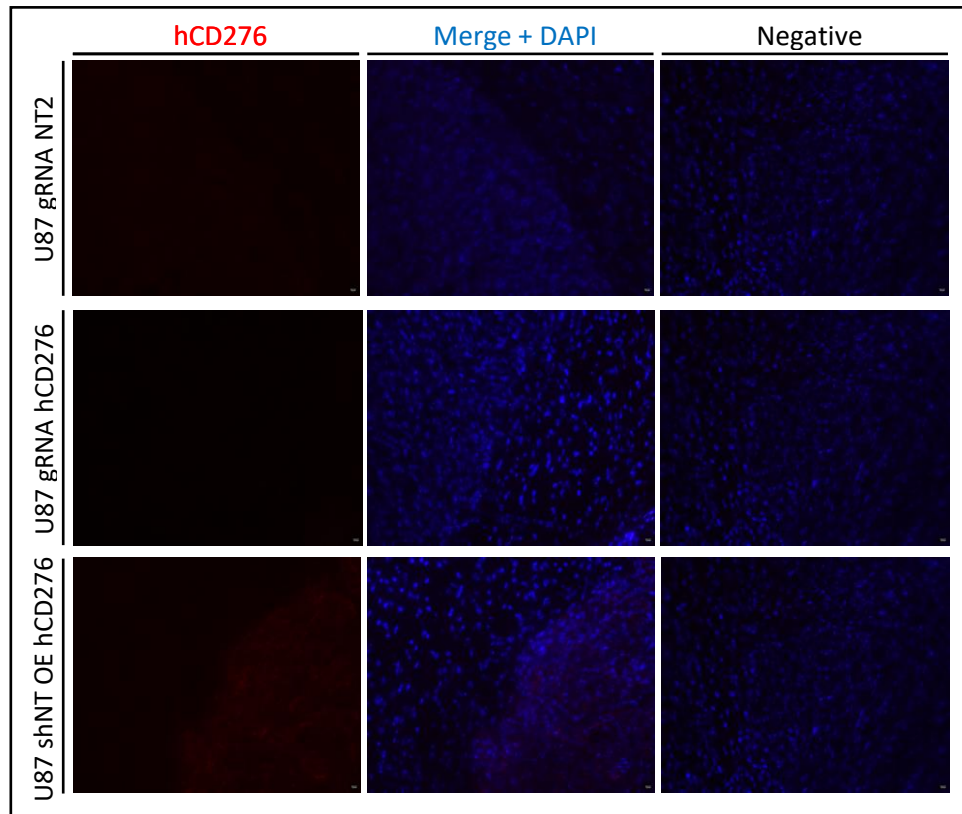
Figure 7. Immunofluorescence on mice brains injected with U87 gRNA NT2, U87 gRNA hCD276 KO and U87 shNT OE hCD276 2Ig (**Table 1**. and **Table 2**.).

The immunofluorescence of mice brains which were injected with U87 cell lines with a primary Ab directed against B7-H3 (**Table 1**.) and a secondary Ab coupled to red fluorochrome (**Table 2**.) does not allow the detection of B7-H3 as there is no signal detected or almost none for the overexpressed lineage (**Fig. 7**). It is probably due to a change in B7-H3 expression in the presence of tumor microenvironment. Brains injected with patient-derived GSCs show (**Fig. 8A**), contrarily to **figure 6A**, a diffuse mass with infiltrative cells that were targeted via a human-Vimentin Ab (**Table 1.**) and that are located in many different regions in the brain. By fluorescence, there is no defined tumor core observed through a higher cellularity with DAPI. B7-H3 is detectable in different fluorescence intensity between the different tumors (**Fig. 8B**). However, the signal appears to be present in nuclei rather than in the cytoplasm or at the membrane as it was detected in those cells in culture.

6. Quantification of microglia cells recruited to the tumor in mice brains
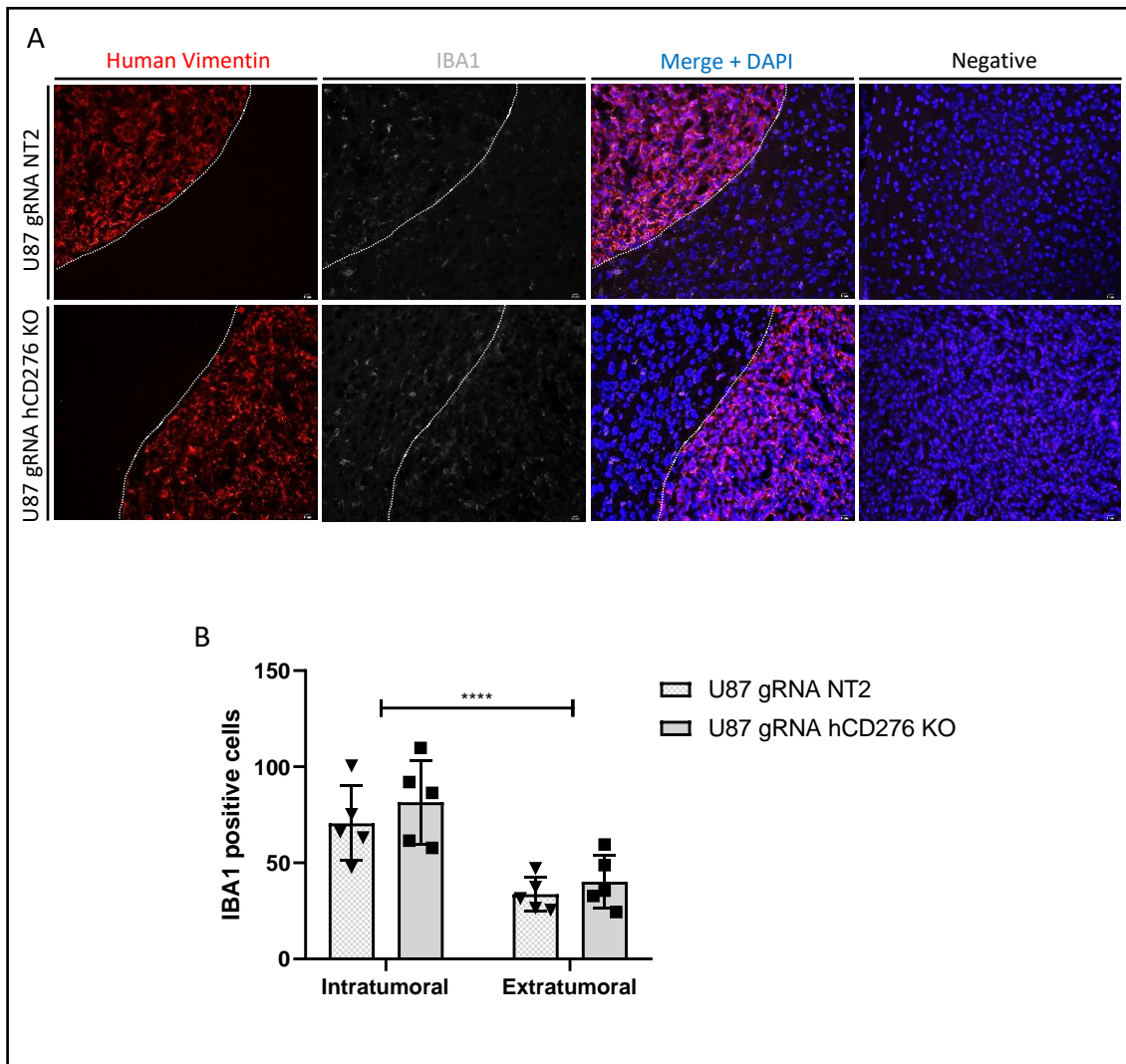
Figure 9. Quantification of IBA1 positive cells in mice brains injected with U87 gRNA NT2, U87 gRNA hCD276 KO and U87 shNT OE hCD276 2Ig. A. Immunofluorescence of tumor core. Antibody anti-human Vimentin (**Table 1**.) coupled to red secondary Ab (**Table 2**.) and an anti-IBA1 Ab (**Table 1**.) coupled to far red secondary Ab (**Table 2**.). B. Counting of IBA1 positive cells inside and outside the tumor mass $p<0.0001$.

Herein, we made an immunofluorescence of microglial cells in order to see immune cells infiltration in tumor mass. Tumor cells were labeled with an anti-hVimentin Ab (**Table 1**.) (**Fig. 9A**). This allows to differentiate IBA1 positive cells inside and outside the tumor core. Microglial cells were counted thanks to an IBA1 labeling via Fiji ImageJ Software. Between tumor cell types expressing or not B7-H3, we observe no significant difference of IBA1 positive cells inside and outside the tumor p=0.2611 ns. However, not considering tumor cell types, there is a significant difference in the number of microglial cells intra- and extratumor (**Fig. 9B**). If we observe again **figure 9A**, we can see a slight increase of IBA1 cells at the border of the tumor.

7. B7-H3 effect on T lymphocytes *in vitro*

Another part of the study is to investigate the role of B7-H3 when expressed in GBM, on the phenotype of T cells (that putatively express B7-H3 unknown receptor(s)). Cultivated T cells are activated by an activator mix (Material and Methods 2.). Before beginning our investigation, it was important to test and validate the concentration of this activating mix to be used. For these T cell experiments, lymphocytes are extracted directly from donors' blood. Donors were adult volunteers from the routine laboratory of Hemato-oncology and from Pr. Rogister's team in GIGA Neurosciences.
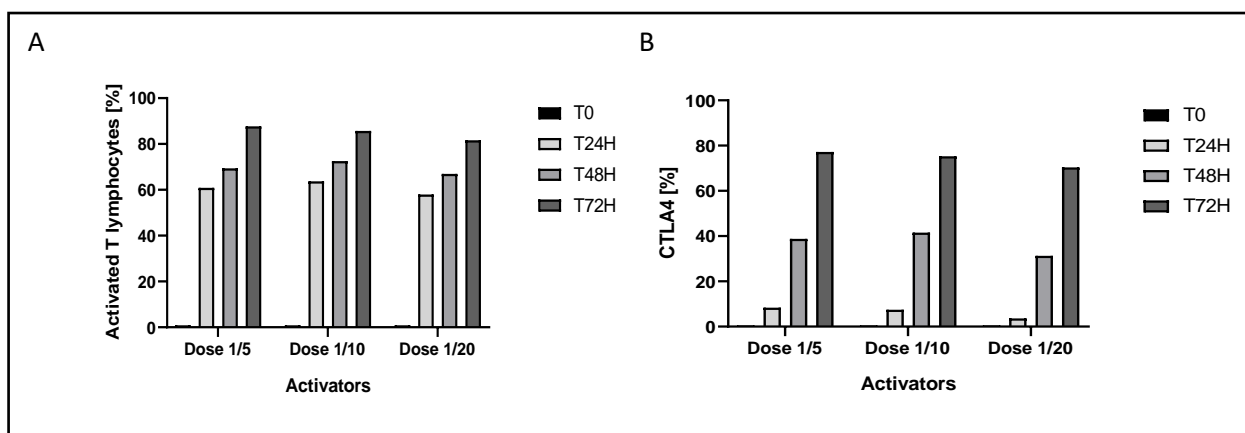
Figure 10. Flow cytometry for different concentrations of activating antibodies. A. Percentage of activated lymphocytes after activation with dilutions 1/5, 1/10 and 1/20 at different timing T0, T24H, T48H, T72H. B. Percentage of CTLA4 immune repressor expressing cells with the different dilutions and depending on the different timing *p*=nd.

To define the concentration of activators to be used, T cell were activated with different activator dilutions and the activation was tested by flow cytometry after 24H, 48H and 72H of incubation (**Fig. 10A**). To properly select activated T cells, there are different important markers (**Table 3.**), i.e., CD45 (for all hematopoietic cells) and CD3 (marker of T cells). To observe the activation loop, we used activation markers as CD69 which is an early activation marker while CD25 is present in late activation. 7AAD is needed to test viability and CD152, also called CTLA4, is important to highlight the presence of a brake in T cell activation as a control for B7-H3 in further analyzes. The CTLA4 increases with time when activation increases (**Fig. 10B**). As there is no significant difference between the activation rate in the different dilutions, we select the dilution 1/20.
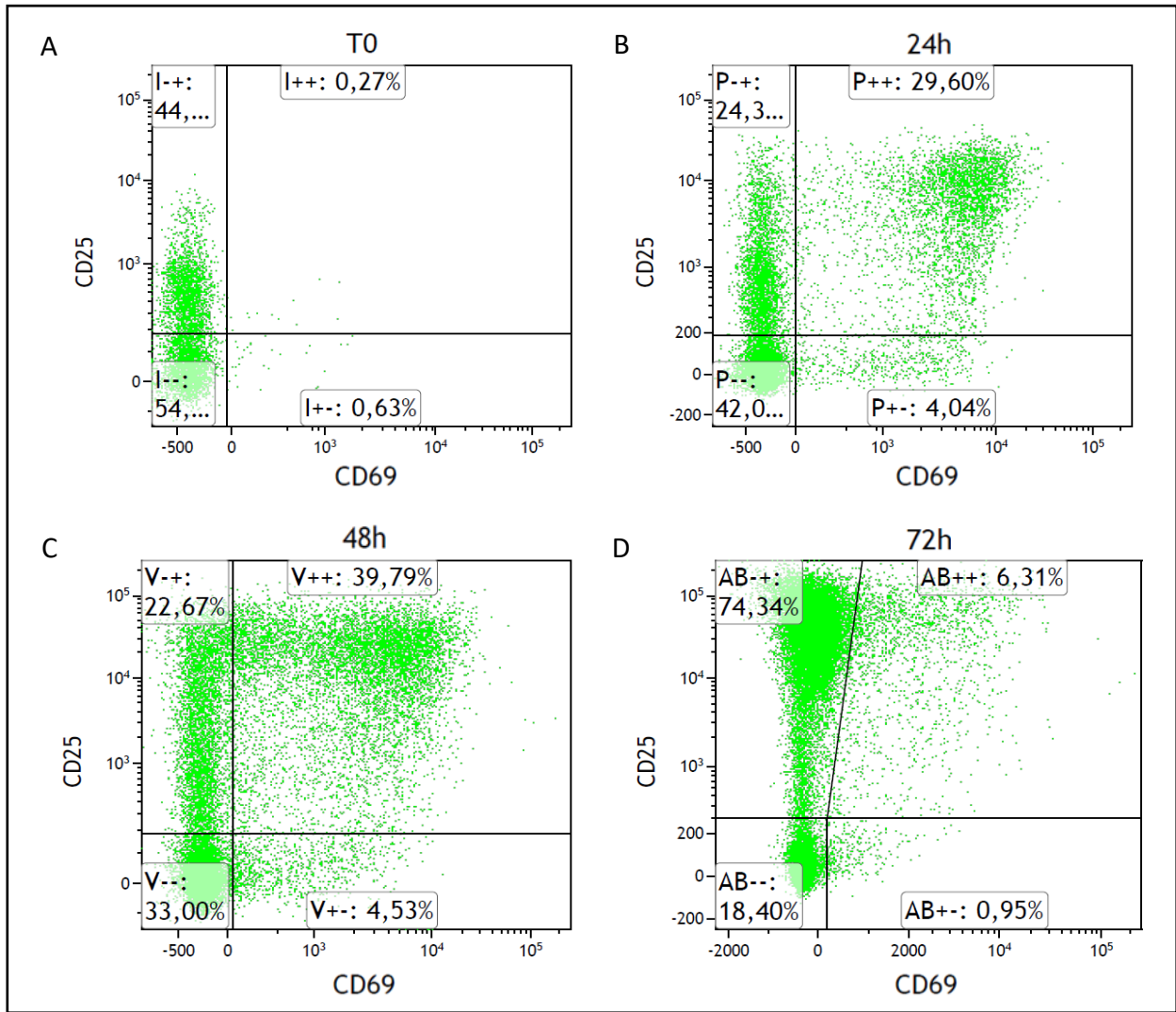
Figure 11. Flow cytometry for analyzing T cell activation at different timing T0, T24h, T48h and T72h. T cells are extracted and activated with dilution 1/20 of activators (Material and Methods 2.).

Thanks to all these previous markers, we are able to target specially T cells and observe the activation loop of T cells in presence of activators dilution 1/20. Before activation, cells are CD25⁻/CD69⁻. When these markers are not detected, there is no activation (**Fig. 11A**). After 24H, the graph shows that the largest proportion of cells is CD25⁺/CD69⁺ ($\simeq$ 85%), cells are in the middle of their activation (**Fig. 11B**). Cells are still CD25⁺/CD69⁺ at 48H (**Fig. 11C**) moving to the third part of the graph in the late activation state CD25⁺/CD69⁻ at 72H (**Fig. 11D**). After validating the activators dilution (1/20) to be used, we begun to optimize the co-culture with U87 glioblastoma cell lines.

7.1.   Optimization of the number of T-lymphocytes

As a first tentative, we put in co-culture $5x10^4$ cells of each U87 cell lines with $1x10^5$ and $2.5x10^5$ T cells for 72H (**Fig. 12A**). By flow cytometry and thanks to all the markers previously cited, we see a difference in the activation state of $1x10^5$ T cells, which is decreased in presence of U87 shNT OE hCD276 2Ig. In contrast, we see a decrease in the activation state of $2.5x10^5$ T cells in presence of U87 gRNA hCD276 KO. Activation was measure before activation (T0 D0) as a control. The difference in the results would probably be due to a lack of precision in the protocol and a lack of reproducibility between the experimental steps. However, we decided to go further with the next steps of optimization since the number of T cells might have to be optimized based on other parameters.

7.2.   Determination of the co-culture duration

In the second experiment (**Fig. 12B**) we co-cultivated $2.5x10^5$ T cells with $1x10^5$ U87 cells of each cell lines. Here, T cells are activated for 48H, and several washes are done to remove all activators before co-culture with U87 cells. The activation state was measured by flow cytometry before activation (T0 D0) and after activation but before co-culture (T0 D2). Then, we measured the activation of T cells in after 4H, 24H and 36H of co-culture. The graph shows that there appears to be a decrease in T cell activation when co-cultured for 4H with U87 shNT OE hCD276 2Ig culture. We hypothesized that (1) the number of T cells could have been too high for observing a significant inactivation and that (2) the effect on T cell activation may occur at 4H of co-culture, even before.
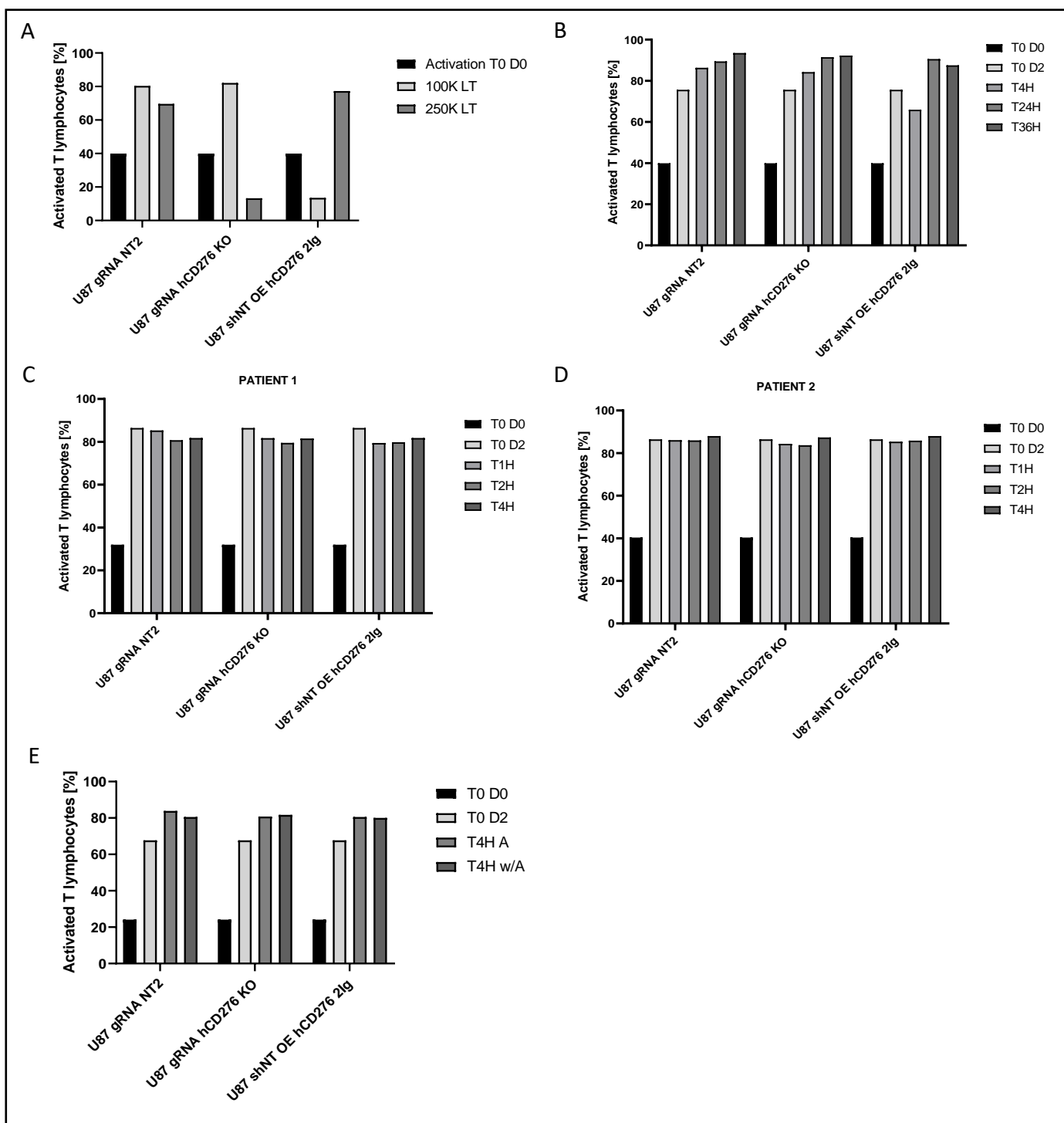
Figure 12. Flow cytometry of T cells in co-culture with the three U87 cell lines gRNA NT2, gRNA hCD276 KO and shNT OE hCD276 2Ig. T0 D0 is the activation state before presence of activators. T0 D2 is the activation state after 48H of activation and in absence of glioblastoma U87 cells. A. $5x10^4$ U87 cells in presence of $10x10^4$ and $25x10^4$ T cells for 72H. B. $5x10^4$ U87 in presence of $10x10^4$ T cells at 4H, 24H and 36H. C and D. $5x10^4$ U87 in presence of $10x10^4$ T cells activated 48H before co-culture in patient 1 and patient 2 at 1H, 2H and 4H. E. $5x10^4$ U87 in presence of $10x10^4$ under agitation (A) and without agitation (w/A) at 4H. p=nd.

7.3.   Determination of the co-culture duration (next)

We co-cultivated $1x10^5$ U87 with $1x10^5$ T cells. T cells were again activated 48H before co-culture. This time, U87 cells were seeded in a plate 24H before. This experiment was repeated with T cells from blood of two different patients/donors (**Fig. 12C and 12D**). The activation state of T cells was analyzed after 1H, 2H and 4H. For both donors, the activation state of T cells was approximately the same for the three timings compared to the control (T0 D2) either in presence of U87 gRNA NT2, U87 gRNA hCD276 KO or U87 shNT OE hCD276 2Ig.

7.4.   Optimization of the U87 culture conditions for co-culture

Our last attempt was to test the same co-culture conditions but to address the impact of U87 cell adherence or suspension during the 4H of co-culture with T cells. Again, the co-culture was done here with $1x10^5$ U87 cells and $1x10^5$ T cells that were previously activated for 48H. T cell activation state was thus measured at 4H under agitation (A) (U87 cells stay with T cells in suspension) and without agitation (w/A) (U87 cells adhere) (**Fig. 12E**). Unfortunately, there is again no difference between both conditions in T cell activation, and in the presence of the three U87 cell lines. Altogether, the potential effect which we have observed once (decreased T cell activation after 4H co-culture with U87 shNT OE hCD276) could not be reproduced.

Discussion

In this master thesis, our objective was to decipher the role of B7-H3, which is a B7 family protein expressed in glioblastoma cells, mainly by glioblastoma cells which exhibit some characteristics of tumor-initiating cells. It acts in different ways in various diseases, such as solid cancers, hematologic cancers and infectious pulmonary diseases. Its checkpoint role is, at least so far, very controversial and that is what we wanted to analyze here. Indeed, as the expression of B7-H3 is found in numerous types of solid cancers, it may explain the heterogeneity of its activity depending on cancer types (S. Liu et al., 2021).

The first step of our research was to confirm *in vitro* the presence of B7-H3 in three U87 cell lineages built to characterize the B7-H3 role(s) in GBM: U87 gRNA NT2 (or wild-type expressing a mid-level of B7-H3), U87 gRNA hCD276 KO, U87 shNT OE hCD276 2Ig. They served either as control to validate our techniques or to test B7-H3 role. Indeed, in immunostainings and flow cytometry experiments, those cells allowed us to look for the presence of B7-H3 in patient-derived glioblastoma stem-like cells that is mainly located at the membrane or in the cytoplasm. In these patient-derived cells cultured as neurospheres, the immunostainings and flow cytometry assays reflected the intertumoral heterogeneity of glioblastoma. Indeed, fluorescence intensity showed that the protein is expressed at different levels in T08, T013, T018 and T033. However, the percentage of positive B7-H3 signal cells observed by flow cytometry showed us a similar number of cells expressing our protein. This suggests that the percentage of cells expressing B7-H3 does not differ depending on the cell lineage but that patient-derived B7-H3-positive cells express it in different proportions in each tumor.

In vivo, we detected the protein in patient-derived GSCs when injected in mice brains, but not in brains injected with U87 cell lineages expressing B7-H3. As B7-H3 acts differently depending on the disease, its expression might be modulated differently depending on the environment. The microenvironment of a tumor such as glioblastoma could regulate negatively the expression of the protein. In brains injected with patient-derived GSCs, we see that the intensity of expression does not vary but we find signal in the nuclei in addition to the membrane signal. This could also be due to an influence of the microenvironment.

The soluble form of B7-H3 (sB7-H3), which was previously described as a transmembrane protein, has been first identified by W. Chen as a novel isoform of the protein lacking the transmembrane domain. On one hand, sB7-H3 seems to be produced by alternative splicing and would thus be secreted in the extracellular medium via exosomes, what is observed in medulloblastoma (W. Chen et al., 2013; Purvis et al., 2020). On the other hand, G. Zhang identified a sB7-H3 that is released from the surface of the cell, shed from the transmembrane form, what is also observed in idiopathic pulmonary fibrosis (G. Zhang et al., 2008; Fang et al., 2022) and in case of osteoporosis, cleaved by MMP-2 (Feng et al., 2016). Moreover, sB7-H3 is present in healthy blood (Jiang et al., 2013), but has been found to be more abundant in patients suffering from ovarian cancer (Deng et al., 2021), hepatocarcinoma, respiratory diseases such as mycoplasma pneumonia (Z. Chen et al., 2013) or diabetes (Fang et al., 2018), among other diseases. This soluble form seems to be active on T cell regulation (J. Xu et al., 2006) and has also been found for the first time in 2014 in CSF to assess sB7-H3 in gliomas patients (Baral et al., 2014). However, the concentration of the protein in the CSF has not been linked to the amount of the membrane form expressed by the tumor cells in this study. The authors proposed that sB7-H3 could be a biomarker for glioma.

To look for the presence of this isoform (sB7-H3) in glioblastoma, we herein investigated both U87 cell lines and patient-derived glioblastoma stem-like cells by ELISA. We observed in different concentrations of sB7-H3 in the culture medium conditioned by build U87 cell lines but also by patient-derived cells when those cells are cultivated as spheres, a condition known to increase the GSCs proportion. It reflects again a certain heterogeneity. There is no certitude of the origin of the soluble form in this case as we know that it could be either secreted as an isoform or released from the cell surface. The sB7-H3 concentration we measured, depending on the lineage, is not correlated to the membrane B7-H3 intensity observed in immunostainings/flow cytometry assays, contrarily to what is observed in gastric adenocarcinoma (W. Chen et al., 2013; L. Huang et al., 2022). Therefore, it could suggest that secretion would be more plausible than shedding. Anyway, it might be interesting to investigate the presence of the sB7-H3 form in CSF in models of glioblastoma xenografts and compare it to the membrane isoform of the protein on the surface of GBM cells. Further investigation may be useful to understand the role of the soluble form compared to the known role(s) of the membrane isoform of B7-H3. Finally, as in U87 shNT OE hCD276 2Ig, there is

only a 2Ig isoform of B7-H3 which is expressed, we can conclude if sB7-H3 is released in the medium by shedding, the 2Ig isoform is thus able to be cleaved. However, it doesn't rule out that the shedding could also be observed for the 4Ig isoform.

B7-H3 is involved in a wide range of diseases such as osteoporosis, respiratory diseases as cited above but it may also be implicated in diabetes or obesity through metabolic regulation (Picarda et al., 2022). However, as previously said, it is known to be also overexpressed in different cancers. It enhances tumorigenesis, proliferation, migration, angiogenesis, etc. (Lai et al., 2019) that altogether, increase tumor progression in solid cancers (G. Zhang et al., 2008). For example, in gastric cancer, B7-H3 promotes cell migration and tumor invasion (Li, Yang, et al., 2017) while it is linked to an anti-apoptotic effect in renal cancer (S. Zhang et al., 2019) that also leads to cell invasion. In addition, in other cancers such as myeloma or bladder cancer, it has been proved to be involved in proliferation or migration and invasion respectively, through the activation of various intracellular signaling pathways (Li, Guo, et al., 2017; Lin et al., 2019). Globally, all those mechanisms are involved in tumor progression and might all be present in a certain way in all cancers, such as in ovarian cancer (J. Zhang et al., 2017).

As the role of B7-H3 appears to be different depending on the cancer, it is thus interesting to determine what are the roles of B7-H3 in glioblastoma. In this study, we investigated tumor cell proliferation *in vitro* and tumor growth *in vivo* using U87 cell lines expressing B7-H3 at basal level (U87 gRNA NT2) and U87 knocked-out for B7-H3 (U87 gRNA hCD276 KO). The proliferation was evaluated by an EdU proliferation assay. This experiment was repeated four times. After consulting the results, the laboratory did not noticed significant differences between the percentage of proliferative cells in U87 gRNA NT2 and U87 gRNA hCD276 KO cell lines, meaning that the protein has no effect in U87 cell proliferation *in vitro*.

For the same purpose, we tested *in vivo* if B7-H3 has an impact on tumor growth. As we used brains injected with U87 cell lineages for this experiment and did not detect the protein of interest in our immunostainings as seen above (**Fig. 7**), it may explain the negative results. At day 0, the three U87 cell lineages are injected in nude mice. These mice were sacrificed, brains were recovered and used for tumor quantification via immunohistochemistry. Tumor size is not different from a cell line to another. This was confirmed after quantification thanks

to *Mercator* (Explora Nova) Software allowing us to reconstitute the tumor in 3D, giving an estimation of the tumor size. The similar tumor volume in presence and in absence of B7-H3 indicates there is no impact of B7-H3 on tumor growth. Please note that mice implanted with U87 overexpressing B7-H3 had to be sacrificed one week before the two other groups, because of these mice were meant to be used for a parallel experiment.

What is disconcerting is that both *in vitro* and *in vivo* studies for investigating the role of B7-H3 on tumor progression gave us results that are different from what other researchers obtained in similar experiments (Lemke et al., 2012; Zhong et al., 2020). In these studies, proliferation kits are different than those used in our experiments and could be the cause of a difference in results. Also, the cell lineage was different in our case and may confer different basis for such investigations. Finally, in overexpressing U87 cells, we only expressed the 2Ig isoform and not the 4Ig. This could also be a reason explaining some discrepancy between our results and results already published about the possible role(s) of B7-H3 in the GBM.

In different studies concerning myeloma or bladder cancer as cited before (Li, Guo, et al., 2017; Lin et al., 2019), or again in lung adenocarcinoma, these effects of B7-H3 on tumor progression have been associated with the activation of intracellular signaling pathways. Y. Li, G. Guo, and their team demonstrated that an overexpression of the protein is involved in the activation of PI3K/Akt pathway (Li, Guo, et al., 2017). The activation of an intracellular signal for proteins belonging to the B7 family such as B7-H3 is possible by binding a receptor from CD28 family. Thus, to activate such pathways, signals and their resulting mechanism in cell survival or growth, B7-H3 should bind a receptor. To confirm the results of these studies, it could be interesting to further identify the B7-H3 receptor and then, to investigate the B7-H3 activation of various signaling pathways after binding to its receptor.

Glioblastoma is a cold tumor in a sense that this tumor is able to shut down the immune reaction around the tumor cells. These "cold" tumors are often characterized by a limited T cell infiltration and activation, a lack of tumor antigens, and defect in antigen presentation (Gajewski et al., 2017). These dysfunctions are resulting from various immunosuppressive mechanisms. One of these mechanisms implicates glioma-associated macrophages (*GAM*). GAM are brain resident macrophages also known as microglial cells, that are able to

differentiate in pro-inflammatory or anti-inflammatory macrophages. In a cold tumor such as glioblastoma, microglial cells differentiate into anti-inflammatory macrophages producing cytokines and chemokines that favor tumor progression through preventing the immune system destructive response of tumor cells. Microglia seems indeed to favor GBM progression (Geribaldi-Doldán et al., 2021).

This aspect of cold tumor is thus interesting considering that B7-H3 is known as an immune checkpoint. For this reason, it could play a role in this immunosuppressive environment. To assess the different mechanisms B7-H3 could be involved in, we investigated the presence of microglial cells in and out of the tumor. Microglial cells are antigen presenting cells (*APC*) and should thus express, as well as T cells, B7-H3 receptor (Flem-Karlsen et al., 2020). We observed that microglial cells are present in higher amounts inside the tumor mass, suggesting their role in the immunosuppressive environment. However, there is no different in the IBA1^{+} cells in presence or in absence of B7-H3 meaning that our immune checkpoint probably does not play a role in the creation of an immunosuppressive microenvironment through the recruitment of microglial cells. Our observation does not rule out that it could play a role in microglial activation. Other immunostaining using M1 and M2 markers in GBM induced by B7-H3 positive or negative tumors could answer to that that question. However, as demonstrated by Bettinger et al., other effects allowing the earlier migration of glioma cells is possible due to microglia (Bettinger et al., 2002). Further investigation is required to understand the role of microglia in the migration of tumor cells with a higher expression of our protein of interest.

Another characteristic of the tumor microenvironment of a cold tumor is the absence of T cell infiltration and activation. As an immune checkpoint, B7-H3 binds to its putative receptor possibly located on T cells, DCs or APCs to regulate the activation of T cells, either positively or negatively. Nowadays, no affirmation of this positive or negative regulation is known. M. Hashiguchi showed that TLT-2, a putative B7-H3 receptor, was activating T cell immune response (Hashiguchi et al., 2008). This was challenged one year later by J. Leitner and his team, who indicated there is no evidence of the binding of B7-H3 to TLT-2 and rather showed a dose-dependent effect of the protein on T cells (Leitner et al., 2009). However, TLT-2 receptor of B7-H3 discovered in 2008 was recently confirmed by C. Fang in idiopathic pulmonary fibrosis (Fang et al., 2022).

To investigate the role of the protein on T cell regulation in glioblastoma, we co-cultivated various U87 cell lines in presence of activated T lymphocytes, directly extracted from donors' blood. Our hypothesis was that, if B7-H3 bound its receptor located on T cell surface, it would inactivate them, and further prevent their activity against tumor cells. Unfortunately, that is not what we observed. In our results, we did not clearly demonstrate a direct effect of B7-H3 on T cell regulation. In the study of A. Chapoval, the role of B7-H3 was shown to activate T cell and elicit an immune response (Chapoval et al., 2001). Here, when we artificially activated T cells, we could not observe such an effect. As seen in 2004 by D. Prasad and his lab, B7-H3 is considered as a negative T cell regulator (Prasad et al., 2004). Nevertheless, they measured the proliferation rate of T cells and not their activation level, rather aiming to identify a general effect of B7-H3 on T cell division. Also, in gastric cancer, D. Ulase and collaborators showed a decrease in T CD8+ density inside the tumor when tumor cells express B7-H3 (Ulase et al., 2021). In contrast, they didn't investigate a coculture experiment but a simple counting in immunostainings. Their approach doesn't rule out an indirect effect of B7-H3 on T cells, involving another cell type, stimulated by B7-H3 and directly responsible of an effect of T cells.

In other publications, the association of B7-H3 with an anti-cancer immune response was more specific. In the study of Y. Jin , they tried to associate B7-H3 amount to the amount of Treg present inside the tumor mass (Jin et al., 2015). This correlation would explain a negative regulation of B7-H3 on T cells through Treg. This would also mean that B7-H3 may be responsible for the activation of T cells and their differentiation in a preferential type of lymphocyte. Indeed, W. Suh showed a downregulation of T helper type 1 (*Th1*)-mediated immune response for the benefit of T helper type 2 (*Th2*)-mediated immune response, which leads, *in fine*, to the secretion of different cytokines that inactivate an anti-tumoral immune response, making Th2 pro-tumorigenic cells (Suh et al., 2003; Basu et al., 2021). We also know that the unknown receptor of B7-H3 should be expressed by NK cells, and that it inhibits NK cells lysis of tumor cells either in ovarian cancer (Deng et al., 2021) or in neuroblastoma (Castriconi et al., 2004). Considering these observations, it might be interesting to think widely to another way of testing, once again taking into account the various cells participating in the anti-tumor immune response.

Conclusion and perspectives

Primarily, the presence of B7-H3 in different concentrations in the different patient-derived glioblastoma stem-like cell lineages is encouraging for research. Indeed, it confirms a heterogeneity of protein concentration but also demonstrates a minimal concentration in patient-derived tumors. When measuring the involvement of the protein in processes such as cell proliferation or tumor growth in nude mice, we can say B7-H3 does not play a role in these mechanisms in our preclinical model. To be as accurate as possible and to ensure the validity of these results, the experiments could be repeated in the presence of cells expressing the B7-H3 receptor. This experiment implies the B7-H3 receptor identification. Moreover, B7-H3 is a transmembrane protein binding a receptor, it might be interesting to test its implication in these mechanisms of cell proliferation and tumor growth in presence of cells expressing the receptor, such as T cells, thus in another mice model. The activation of the ligand-receptor couple may activate different intrinsic pathways and generate an activation or deregulation of the underlying pathways involved in growth factor transcription.

In the context of this master thesis, coculture experiences for assessing the effect of B7-H3 on the activation of T cells expressing the receptor of the protein did not show any positive results. B7-H3 expressed by GBM cells might not have this direct effect on lymphocytes. As previously said, the binding of B7-H3 on its receptor on active T cells could also activate an intrinsic pathway in glioblastoma cells which ultimately acts on a DNA regulation mechanism impacting various cell survival or cell death processes. Looking at microglia cells, we observed a higher amount of these cells into the tumor than outside. However, no difference was observed in absence or in presence of B7-H3. Even if the presence of microglia cells seems to not be due to the presence of the protein, B7-H3 is possibly involved in the creation of an immunosuppressive microenvironment. Indeed, the vicinity between microglia and glioblastoma cells may induce a differentiation of microglia into type 2 anti-inflammatory macrophages and lead to an environment that support tumor growth rejecting immune cell infiltration.

To conclude, despite the controversial role of B7-H3 in various cancers, it is interesting to study further the different intrinsic and extrinsic role of this protein on the potential activation of intracellular signaling pathways in GBM. The investigation of these roles in GBM may indeed allow to understand the mechanisms involved in this cancer and how B7-H3 acts on the GBM rapid progression and/or recurrences. Understanding these mechanisms of action will allow the protein to be considered as a real therapeutic target in glioblastoma.

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Annexes
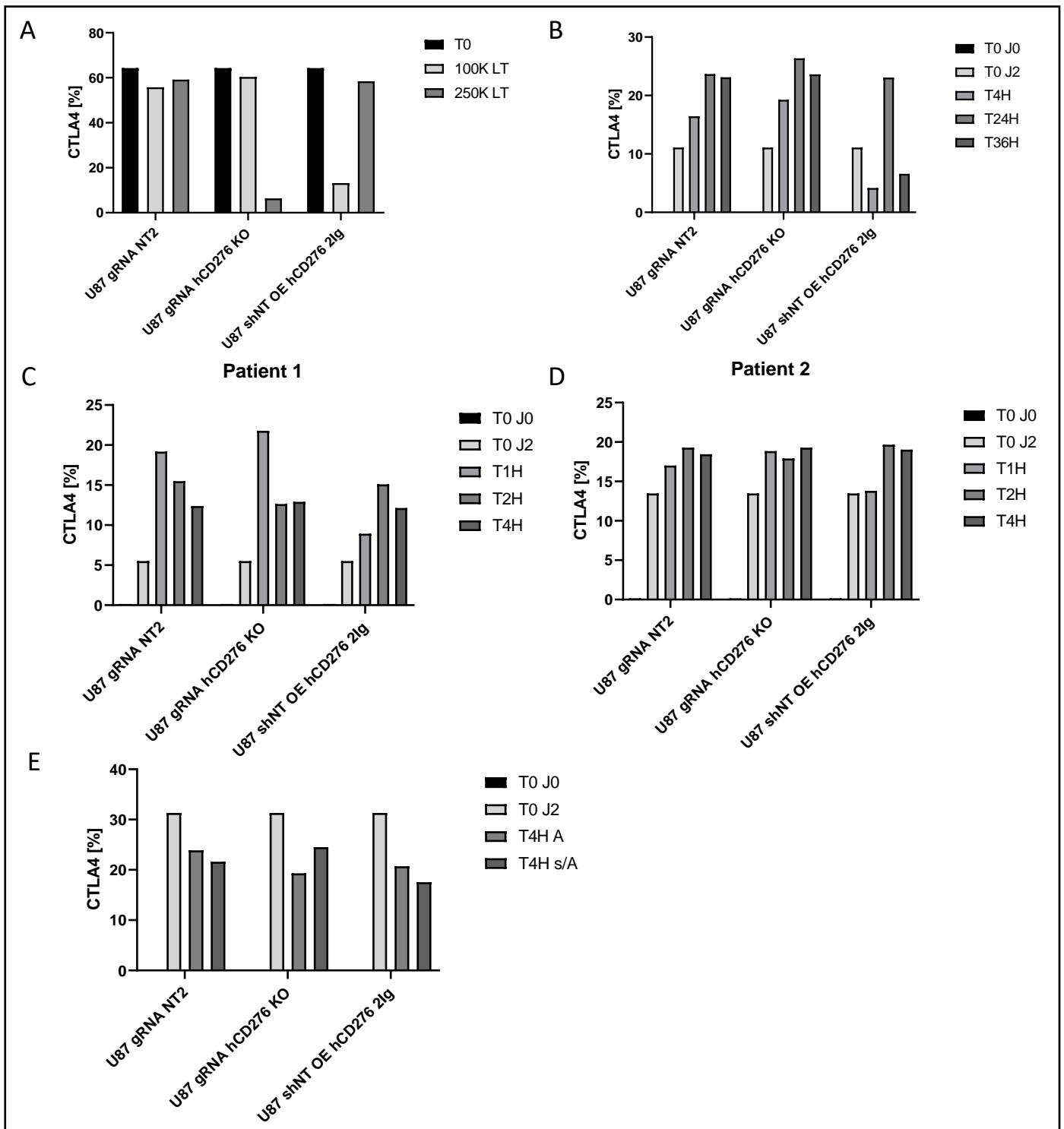
Annex 1. Flow cytometry of T cells in co-culture with the three U87 cell lines gRNA NT2, gRNA hCD276 KO and shNT OE hCD276 2Ig. Measure of CTLA4 presence [%] for assessing the negative regulation of T cell regulation. A. 5×10^4 U87 cells in presence of 10×10^4 and 25×10^4 T cells for 72H. B. 5×10^4 U87 in presence of 10×10^4 T cells at 4H, 24H and 36H. C and D. 5×10^4 U87 in presence of 10×10^4 T cells activated 48H before co-culture in patient 1 and patient 2 at 1H, 2H and 4H. E. 5×10^4 U87 in presence of 10×10^4 under agitation (A) and without agitation (w/A) at 4H. p=nd.