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Immuno-Radiotherapy for brain glioma: sorting out the immunomodulatory effects of radiotherapy

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A Cucciola Lili Ulisse e Niño

Alla gioia, al coraggio, alla resilienza

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Glioblastoma

Glioblastoma (GBM), which is a World Health Organization (WHO) grade IV tumour, is a fast-growing and aggressive brain tumor. It invades the nearby brain tissue, but generally does not spread to distant organs.

GBMs can arise in the brain de novo or evolve from lower-grade astrocytoma. In adults, GBM occurs most often in the cerebral hemispheres, especially in the frontal and temporal lobes of the brain. GBM is a devastating brain cancer that can result in death in three-six months, if untreated; hence, it is imperative to seek expert neuro-oncological and neurosurgical care immediately, as this can affect overall survival.

GBM is the most frequent malignant primary brain tumour. It is characterized by microvascular hyperplasia, necrosis and/or specific molecular characteristics, including TERT promoter mutations, EGFR gene amplification, and/or a + 7/- 10 cytogenetic feature [1]. Despite continuous progress in glioma diagnostic and therapeutic strategies, the morbidity and mortality rate of glioma remain high.

This high morbidity is associated with high tumor heterogeneity; early and widespread diffuse malignant cell infiltration, difficulty in achieving complete surgical removal, and high intrinsic chemotherapy and radiotherapy resistance [2].

Historically, glioma classification was based on histological and immunohistochemical criteria. Despite the low impact of molecular classification in medical diagnosis, during the past few years, there have

been remarkable advances in this field, especially for the central nervous system (CNS) tumor classification, which was included in the fifth edition of the WHO Classification of Tumors of the Central Nervous System, published in 2021 [3]. This new edition integrates molecular changes with clinicopathological utility essential for accurately classifying CNS tumors. This edition also introduces changes to the former taxonomy and nomenclature, including the term "type" instead of "entity" and "subtype" instead of "variant."

The 2021 WHO Classification of CNS Tumors defines three genetic parameters for diagnosing glioblastoma, IDH-wildtype: TERT promoter mutation, epidermal growth factor receptor (EGFR) amplification, and the combined gain of entire chromosome 7 and loss of entire chromosome 10 [4].

Treatment strategies for GBM rely on inducing DNA damage. The current standard of care (SOC) therapy consists in maximal safe surgical resection followed by radiation therapy and adjuvant temozolomide (Stupp protocol) [5], with a median overall survival (OS) of 8-10 months. However, more than half of GBM patients die within one year from the diagnosis, and only 5% survive more than 5 years despite aggressive therapies. GBM treatment remains dismal even though great progresses in the management of the pathology.

Understanding the molecular characteristics of GBMs has improved our knowledge of the disease course, response to treatment and allowed for prognosis [6].

GBMs are now accepted as either primary or secondary, both of which are histologically indistinguishable from each other [7]. Mutations in the genes that code for the enzyme isocitrate dehydrogenase, *IDH1* and

less commonly *IDH2,* (a key enzyme in the tricarboxylic cycle and glutamine metabolism) now unequivocally define a secondary GBM, [Yan H 2009] whereas the *IDH*wild type is considered primary GBM that arose de novo as a higher-grade tumour. *IDH* mutated secondary GBMs were lower-grade gliomas that eventually underwent a malignant transformation [8].

These secondary GBMs have a far better prognosis with longer median survivals than *IDH* wild-type primary GBMs [9]. Equally important is O^6-methylguanine-methyltransferase (*MGMT*) gene silencing by methylation.

Approximately 50% of all newly diagnosed GBMs are *MGMT* methylated [10].

The *MGMT* gene found on chromosome 10q26 codes for the MGMT protein, a DNA repair enzyme [11]. This protein removes alkyl groups from guanine nucleotide at the O6 position, which is thought to be the site of action of TMZ. Silencing reduces MGMT protein expression leading to decreased DNA repair, rendering these patients more sensitive to TMZ and significantly prolonging survival than unmethylated patients [12].

Thus, in addition to age, preoperative performance status, the extent of tumour resection, molecular characteristics such as *IDH* mutations and *MGMT* methylation are recognized as independent prognostic factors affecting overall survival [12].

Despite being the treatment approach well defined, recurrence is inevitable, due to GBM infiltrative behavior. Unfortunately, there is no standard treatment for recurrent GBM.

1.2 CNS and GBM: immunological features

The intrinsic cold tumor immune microenvironment (TME) in gliomas, characterized by a high ratio of pro-tumor to anti-tumor immune cell infiltrates, acts as a seemingly insurmountable barrier to immunotherapy.

The parenchymal inflammatory cells are represented by astrocytes and microglia-derived macrophages [13]. Astrocytes are the first host cell type encountered by cancer cells after extravasation. As soon as cancer cell extravasation starts, astrocytic activation has been shown to be effective [14], characterized by GFAP-positive cells (GFAP: glial fibrillary acidic protein). This activation is known to be able to liberate MMP9 near cancer cells, releasing proangiogenic and growth factors from the extracellular matrix promoting tumour cell proliferation [15]. An accumulation of microglial cells in peritumoural and perinecrotic regions, predominantly macrophages, forms a microglial wall whose density varies nevertheless between primary tumour types (Figure 1). Interestingly, peritumoural microglial cells expressed CD68 and CD163 in a higher proportion than control regions, which are scavenger receptors implicated in phagocytosis. In contrast, enzymes involved in oxygen and nitric oxide radical production were unexpressed or weakly expressed, attesting to potential brain-sparing behaviors of the microglia-derived cells [16].

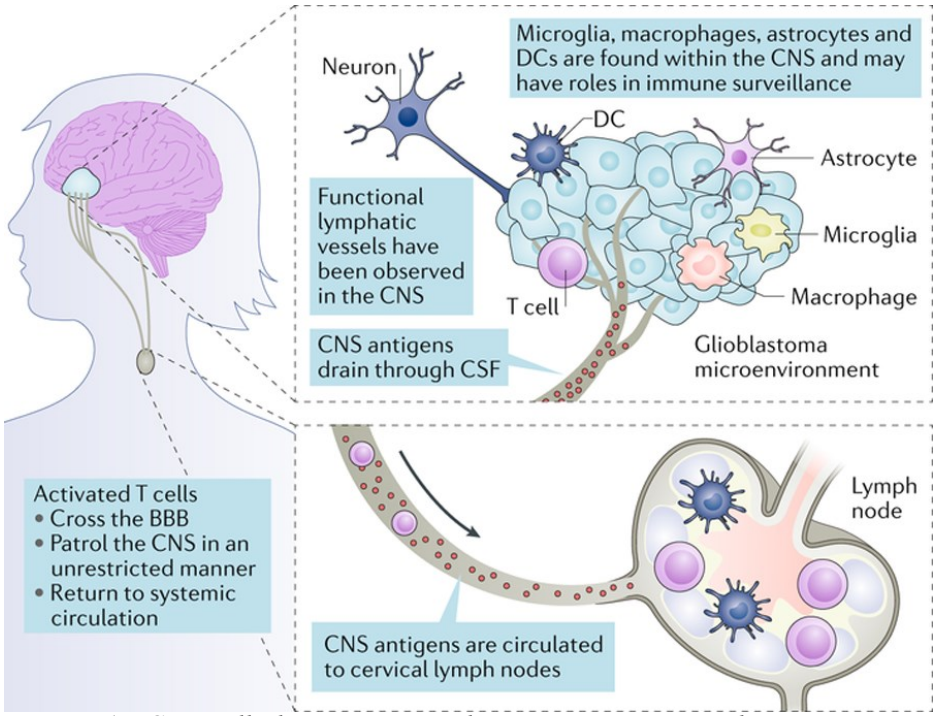

Figure 1. Crosstalk between central nervous system and immune system (Sampson, Nat. Rev. Cancer 20, 12–25).

The innate immune system, which constitutes our first line of defense, mediates broad responses against pathogens while also activating adaptive immunity for more specific targeting. Research has now shifted additional attention to methods of modulating the innate immune system for the treatment of GBM.

The role of the innate immune system for GBM therapies are related to Tumor-Infiltrating Myeloid Cells, NK Cells, Gamma Delta T Cells and B cells, which are involved in both the adaptive and innate immune systems. Tumor-infiltrating myeloid cells are the most abundant cellular infiltrates in GBM, which can sometimes comprise up to 50% of the tumor mass [17].

Natural killer (NK) cells are characterized by the expression of CD16 and CD56 surface antigens and lack CD3/T-cell receptor molecules. In contrast to T cells, NK cells do not require antigen sensitization prior to killing targets [18]. Although NK cells can migrate into the GBM microenvironment, the tumor-infiltrating NK cells are significantly altered and their cytotoxic function become impaired, allowing glioblastomas to evade NK cell targeting. Both Gamma delta T cells and B cells play a role in both the innate and adaptive systems. Gamma delta T cells have a cytotoxic effect on GBM cells [19], while B cells that express the co-stimulatory marker 4-1BBL, are associated with decreased T-cell death, enhanced T-cell proliferation, and improved immunological memory in GBM.

Both intrinsic characteristics of cancer cells and extrinsic interactions within the sophisticated tumor microenvironment (TME) contribute to treatment resistance and tumor aggression [20] (Figure 2).

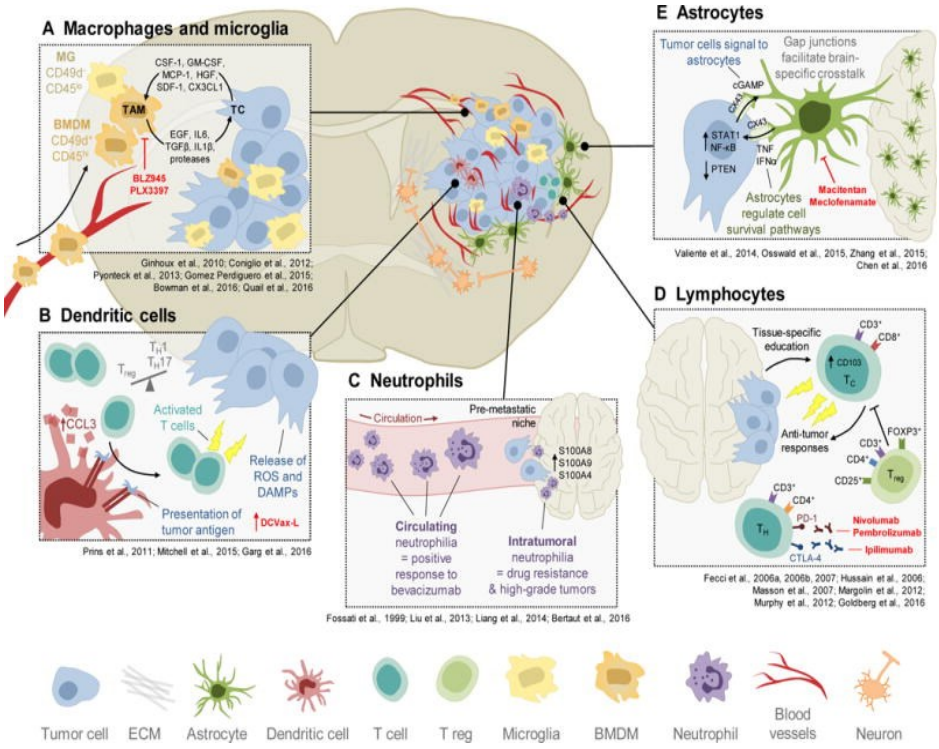

Figure 2. Immune and brain components composing the Tumor Microenvironment (TME, Quail et al. Cancer Cell 2017, 31, 326–341.

Macrophages are the main actors of tumor-promoting inflammatory signals, and a high density of tumor-associated macrophages (TAMs) infiltration is associated with high-grade tumors and a dismal prognosis [21].In fact, a hallmark of GBM and significant barrier to immunotherapies is the immunosuppressive TME defined by relatively high numbers of suppressive, pro-tumor immune cell infiltrates (e.g., regulatory T cells (Tregs), tumor-associated microglia, tumor-associated macrophages (TAMs), myeloid derived suppressor cells and high prevalence of dysfunctional T cell states (e.g., exhaustion) [22] (Figure 3). High infiltration of immunosuppressive myeloid and

lymphoid cell populations negatively correlates with patient prognosis and therapy response in GBM

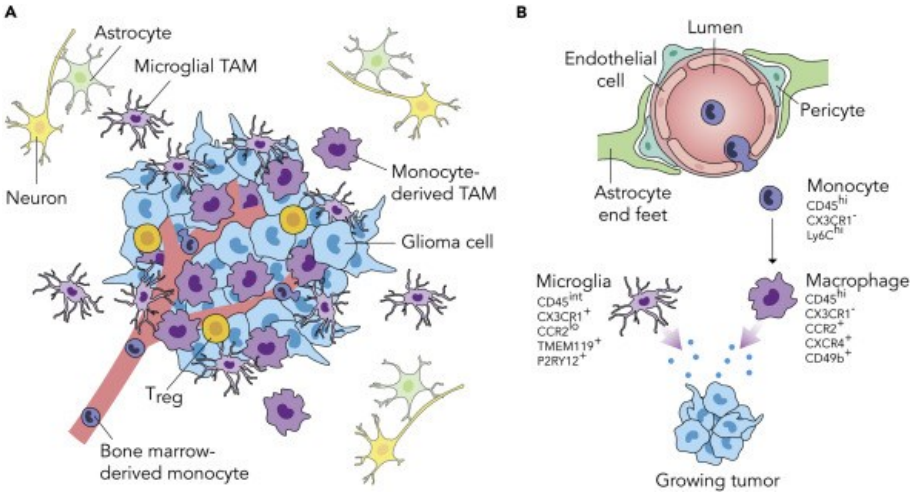

Figure 3. Myeloid derived cells infiltrating tumor mass (Hashimoto et al, Annu. Rev. Med. 2018, 69, 301–318.

In addition, molecularly distinct gliomas, depending on their IDH mutation status, have different immune compositions and landscape that defines its TME [23]. IDH-mutant gliomas are almost totally devoid of TILs in comparison with brain metastasis that are highly enriched with activated and exhausted T cells [24]. This status of low infiltration of T cells in gliomas, especially in the IDH-mutant subtype, creates an environment with low expression of immune checkpoint targets, which provides one possible reason for the resistance to immune checkpoint inhibitors. On the other hand, IDH-mutant tumors are enriched with tumor-resident microglia, which is in contrast to IDH

wild-type and brain metastasis that are infiltrated with monocyte-derived macrophages originating from the periphery [24].

Furthermore, the accessibility of the brain to immunotherapy has been a debate for long with the central nervous system (CNS) historically considered as an immune privileged area thanks to the selective permeability of the blood-brain barrier (BBB), the paucity of specialized APC, and the low expression of MHC-I in brain parenchyma limiting antigen presentation. Nevertheless, recent data have redefined the immunological activities in the CNS. Brain diseases such as autoimmune diseases or infections by neurotropic viruses generate inflammation showing that a robust immune response can occur in the brain. CNS immune surveillance is ensured by resident APC, called microglia that can migrate to inflammatory sites for antigen presentation. Recent works have also identified a lymphatic network, draining the cerebrospinal fluid and meningeal leukocytes, where CNS-derived antigens are captured by APCs and presented to T cells [26]. The secretion of different proinflammatory chemokines and cytokines will compromise the BBB integrity and allow immune cells infiltration [27]. The presence of T infiltrating lymphocytes (TIL) after brain lesions is another argument for cerebral anti-tumor responses.

The GBM vasculature also drives immunosuppression by regulating immune cell function, immunosurveillance, and immune cell trafficking [28].

The tumor vasculature is one of the important components of the TME. The normal structural organization of blood vessels is disrupted in tumors leading to the formation of abnormal vessels which are leaky, collapsed, and disorganized, which contributes to hypoxia, alters tumor

metabolism, tumor invasion, immune suppression, and creates specific niches in TME [29].

Moreover, the erratic vessels induce hypoxia that further stimulates angiogenesis, and abnormal angiogenesis promotes more hypoxia creating a vicious cycle. These events lead to immunosuppression and reduced trafficking of effector immune cells to TME. In fact, it plays a critical role in the establishment of a local hub that supports immunosuppression, hypoxia, and acidosis, escalates interstitial fluid pressure, and makes a physical barrier to T cell infiltration [30]. The vasculature mediates immune evasion and thwarts T cell-mediated immunosurveillance and antitumor immunity in GBM [30].

Overall, the immune landscape of brain tumours is intensely investigated, unveiling new insight in the interactions between neoplastic cells and the immune system [31]. Reciprocal interactions between cancer cells and noncancerous immune and stromal cells in the tumor microenvironment (TME) not only support tumor development and progression but can also contribute to intrinsic and acquired resistance to anticancer targeted therapies [32]. Cells of myeloid origin, as resident microglia and bone marrow- derived macrophages, represent up to one third of the tumor mass and exert pro-tumorigenic functions, sustaining tumor progression and an immunosuppressive TME [33]. In depth investigations of glioma-associated macrophages/microglia (GAMs) and other myeloid cells, as dendritic cells and neutrophils, and their interplays with cytotoxic lymphocytes are needed to improve our understandings on the establishment of the TME and to apply them for innovative therapies against GBM.

1.3 Immunotherapy approaches for GBM

Tumors in the central nervous system (CNS), behind the bloodbrain barrier (BBB), have long been believed to be beyond the reach of the immune system, because the BBB limits trafficking of antigens and immune cells to the CNS. Recent evidence shows intracranial tumor TAAs interact with the peripheral immune system in the cervical lymph nodes. This provides a physiological foundation for testing immunotherapy for intracranial disease.

GBM cells alter the immune system to increase their malignancy. At the same time, in GBM tumors, the BBB integrity is altered due to the damage on endothelial tight junctions revealing molecular composition changes [34]. The BBB breakdown facilitates CD8 + T cells migration to the CNS, as well as innate and adaptive immune responses activation, producing cytokines and chemokines for lymphocyte recruitment and up-regulation of immunomodulatory markers on cells surface [35]. Moreover, RT leads to a direct and indirect damage to tumour cells causing cell deaths, an alteration of the tumour stromal microenvironment and an activation of CD8 + T cells. Radiation induces activation of biological mechanisms and biochemical events, including stimulator of interferon genes (STING) pathway and up-regulation of transforming growth factor β (TGF-β) signaling, triggering immune responses [36].

Simultaneously, the field of innovative immunotherapeutic approaches to treat glioblastoma is rapidly expanding. Numerous current and future directions have been being evaluated:

1. Immune checkpoint regulators, targeting the balance of activating and inhibitory signals on T-cell activation (CTLA-4 and PD-1 or

PD-L1 blockades): Currently a handful of monoclonal antibodies against CTLA-4 (ipilimumab, tremelimumab), PD-1 (nivolumab, pembrolizumab, pidilizumab), and PD-L1 (BMS-936559, atezolizumab, durvalumab) are being tested in a variety of solid tumors. A number of these immune checkpoint inhibitors are also in phase IeIII trials for glioblastoma, with preliminary data forthcoming;

2. Immunostimolatory gene therapies: Oncolytic viral therapy; Cytokine therapy; Suicide gene therapy;
3. Adjuvant therapies: Tumor-associated macrophages (TAMs) therapy;
4. Passive immunotherapy: Chimeric antigen receptor (CAR) T cells; antibodies;
5. Epigenome therapy: Inhibitors of mutant IDH (mtIDHi); EZH2 inhibitors (EZH2i); DNA methylation inhibitors (DNMTi); Histone deacetylase inhibitors (HDACi);
6. Active immunotherapy: Vaccinations (EGFRvIII-mediated vaccine; Dendritic cell vaccines);
7. Combination of different therapies.

1.3.1 Immune checkpoint regulators. Immune checkpoints are important regulators of immune activation, and they play a key role in maintaining immune balance and preventing autoimmune diseases [37]. Activated T cells are the major mediators of immune effector system, and they express multiple co-inhibitory receptors: lymphocyte-activation gene 3 (LAG 3), PD-1 and CTLA- 4, which are used to regulate the responses of tumor antigens. The action pathway is

different for the various immune checkpoint molecules. The main action of PD-1/ PDL pathway is to inhibit the activation and the proliferation of T cells, as well as arrest the production of cytokines. Whereas, the CTLA-4 path causes cell-cycle arrest and apoptosis, in both Tregs and activated T cells [38].

Preclinical studies have shown that systemic effects of RT are mediated by CD8+ T-cell activation [39]. However, hypofractionated RT can induce the expression of checkpoints as the programmed death-ligands PD-L1, PD-L2 and CTLA4 which are master regulators of T-cell activation, both associated with dysfunctional CD8+ T-cells and a profound immunosuppressive microenvironment [40].

Unfortunately, they have had little clinical benefit in GBM, at the least in the adjuvant setting. The recently published results of the open-label, randomized, phase 3 trial CheckMate-143, which evaluated nivolumab vs. bevacizumab in patients with recurrent GBM were disappointing, as there was no significant difference in median overall survival (mOS) between the two arms [41]. The two phase 3 trials CheckMate-498 and CheckMate-548 evaluating the use of nivolumab in patients with newly-diagnosed GBM, either methylguanine methyltransferase (MGMT)-unmethylated or MGMT-methylated, also failed to meet their primary endpoints, according to an update by Bristol-Myers Squibb. Overall, the dismal results in GBM may be due to the poor immunogenicity of GBM tumors.

1.3.2 Immunostimolatory gene therapies: Oncolytic viral therapy; Cytokine therapy; Suicide gene therapy

Oncolytic virotherapy using tumor-lytic viruses can induce an oncolytic cascade. Lysed tumor cells release virions, viral components, and cellular debris encompassing highly immunostimulatory danger- and pathogen-associated patterns (DAMPs and PAMPs), which can serve as a strong induction of immune responses [42]. The clinical evidence of complete remission in a patient treated with oncolytic measles virus (MeV) in relapsing drug-refractory myeloma further is a strong indicator for the oncolytic efficacy of MeV [43].

MeV has already also been clinically tested for the treatment of GBM patients. MeV can be directly re-targeted to typical tumor markers of glioma, such as epidermal growth factor receptor (EGFR) and/or EGFRvIII,15 or even against glioma stem cells [44]. However, MeV is not the only virus species that is developed as an anti-glioma entity, such as convection-enhanced intra-tumoral delivery of recombinant nonpathogenic polio-rhinovirus chimera, a gamma-retroviral replicating vector encoding cytosine deaminase (Vocimagene amiretrorepvec, Toca 511), replication-competent oncolytic adenovirus DNX-2401 (tasadenoturev). Pre-clinical and clinical studies on these virus species showed subgroups of long-term responders. Moreover, sequential triple combination of TMZ (or CCNU), MeV, and RT has synergistic anti-glioma activity, and it leads to an actionable treatment-induced molecular and immunological signature. Some studies showed that a chemo-RT-VT regimen could be combined with tailored peptide vaccinations with our newly identified peptide sequences, potentially in combination with checkpoint blockade antibodies [45].

1.3.3 Adjuvant therapies: Tumour-associated macrophages (TAMs) therapy

The GBM tumour microenvironment (TME) primarily contains tumour-associated microglia and macrophages (TAMs), which constitute up to 30% of the total tumor [46]. TAMs secrete cytokines and growth factors, which lead to or support different biological functions in the TME, such as stemness, proliferation, angiogenesis, cancer cell migration, and immune suppression. Two main TAM phenotypes have been described: the classical, pro- inflammatory M1 TAMs and the alternatively activated, anti-inflammatory M2 TAMs. M2 macrophages have been divided into three subtypes—M2a, M2b, and M2c—with very different functions, such as the involvement in allergy, immune regulation, and tissue remodeling [47]. Easily, M1 TAMs are anti-tumorigenic, secreting pro-inflammatory cytokines, like tumor necrosis factor-α (TNF-α), and mediating Th1 responses. M2 TAMs have pro-tumorigenic functions and secrete anti-inflammatory cytokines—such as transforming growth factor-β (TGF-β)—which inhibit cytotoxic T cells and attract Tregs and MDSCs.

However, TAMs are now considered as cells with high plasticity, which can assume many functions and phenotypes [48]. In addition, populations of TAMs co-expressing M1 and M2 markers have been identified in GBM [49].

Glioblastoma cells secrete several factors that regulate TAM phenotype, survival, and recruitment to the TME. Several factors are involved in the crosstalk between GBM cells and TAMs. Most of these factors either attract and recruit TAMs to the tumor or polarize the TAMs

towards a more pro-tumorigenic M2-like phenotype. Moreover, also extracellular vesicles released by glioblastoma cells play a critical role in tumour progression: GBM cells release extracellular vesicles containing programmed death (PD) ligand 1 (PD-L1) and phospho-STAT3, which can be taken up by TAMs and polarize them towards the M2-like phenotype [50]. Simultaneously, many signaling factors released by TAMs interact with GBM leading to stemness and proliferation. Epidermal growth factor (EGF) receptors (EGFRs) are involved in glioma cell proliferation, and tumor-associated microglia secrete EGF that activates EGFR in glioblastoma cells [51]. In addition, stress-induced phosphoprotein 1 (STIP1) secreted by TAMs induces proliferation in vitro, while IL-10 was shown to promote glioma cell proliferation via JAK2/STAT3 signaling in a glioma model [52]. Additionally, recent results show that TAMs secrete IL-1β, which promotes the rate of glycolysis in glioma cells through glycerol-3-phosphate dehydrogenase (GPD) enzyme [53]. Apart from promoting cancer cells, TAMs also play crucial roles in T cell inactivation and immune suppression leading to tumor immune evasion. TAMs may be part of GBM treatment resistance and RT leads to TAM recruitment to the tumor, especially of macrophages [54]. Moreover, in GBM recurrence a higher macrophage-to-microglia ratio is shown. Furthermore, radiotherapy induces a more M2-skewed TME, which might be explained by increased radio-resistance of M2-like TAMs, as suggested by preclinical studies [55].

TAMs can be therapeutically targeted in different ways: targeting TAM recruitment to the tumor, reprogramming TAM polarization towards a more anti-tumor, M1-like phenotype, or decreasing or eliminating

tumor-promoting M2-like TAMs. TAM recruitment can be prevented by inhibiting the chemokine gradient axes involved in recruiting TAMs to the tumor, such as the CCL2-CCR2 and CXCL12-CXCR4 axes [56]. The most studied approach is increasing the M1/M2 TAM ratio to polarize TAMs towards the pro-inflammatory, anti-tumorigenic M1-like phenotype, and several targets are being investigated—also in the clinic [57].

Lastly, different studies are focused on targeting the M2-like TAMs since a validated marker for macrophages is not known. In fact, tumor-promoting TAMs can be either all M2-like TAMs—including both M2-polarized microglia and macrophages—or macrophages only, since some studies suggest that mainly macrophages are pro-tumorigenic, while microglia are more anti-tumorigenic [58].

However, more research is necessary to proceed along this line.

1.3.4 Chimeric antigen receptor (CAR) T cells; antibodies.

Tumor-targeting CARs are genetically engineered receptors that combine the antigen specificity of antibodies using single chain variable fragments (scFv) with the potent antitumor effects of activated T-cells [59]. However, the use of antibody-derived scFv limits antigen selection to surface bound proteins. Different studies are focused on genetically engineered T-cells expressing a physiological form of tumour antigen-reactive T-cell receptor (TCR) in patients where tumour-specific neoantigens are derived from intracellular proteins [60]. However, genetically engineered T-cell therapy in brain tumour patients has encountered various challenges. Some of these hurdles are shared among all solid tumour types, such as antigen heterogeneity and

tumour-derived immunosuppression, while other challenges are characteristic to CNS malignancies, such as the absence of professional antigen-presenting cells and the limitations to lymphocyte homing resulting from the blood-brain barrier. Despite the complex barriers associated with treating CNS cancers, several early phase CAR T-cell clinical studies provide encouraging data. One of the key challenges that has hindered development of CAR therapies for GBM is the limited availability of targetable tumour-specific antigens which are at the same time safe for normal tissues.

A tumour-specific GBM mutation is variant III of the epidermal growth factor receptor (EGFRvIII). This truncated receptor is expressed in 20% of newly diagnosed GBM patients and has not been found to be expressed on normal tissues [61].

Also, IL-13 receptor α2 (IL-13Rα2) is a promising non-mutant GBM-associated antigen due to its low expression levels in normal brain and it is overexpressed in 75% of GBM patients and it is a prognostic indicator for poor survival [62]. This monomeric high affinity receptor binds IL-13 but not IL-4 and drives the production of transforming growth factor-β (TGF- β) in the tumour microenvironment (TME) [63].

1.3.5 Epigenome therapy: Inhibitors of mutant IDH (mtIDHi); Histone deacetylase inhibitors (HDACi)

Epigenetic alterations modulate cellular phenotype through changes in gene expression without modifying the DNA sequence [64]. The most studied for GBM applications are inhibitors of mutant IDH (mtIDHi) and histone deacetylase inhibitors (HDACi).

Recent studies provided emerging insights into how *IDH* mutations affect the glioma microenvironment. Cytotoxic T lymphocytes (CD8+, cluster of differentiation 8 positive) are crucial components of the tumour-specific adaptive immunity. Lymphocyte infiltration occurs to some extent in glioma, and the presence of tumour-infiltrating lymphocytes (TILs) is predictive of clinical outcome [65]. A recent study demonstrated that *IDH*-wt is associated with the significantly higher TIL infiltration and PD-L1 expression among all grade II–IV gliomas and within the cohort of GBMs [66]. Many preclinical and clinical data validated IDH1/2 as an important target for antitumour drug development. A growing number of studies using cellular and animal models indicate that pharmacological inhibition of mutated IDH1/2 offers therapeutic benefits and there is a rationale for development of isoform-specific inhibitors. In vitro and in vivo preclinical studies demonstrated that inhibition of mutated IDH1/2 enzymes reduces intracellular 2-HG levels, reverses epigenetic deregulation, and releases the differentiation block in cancer cells. These findings provided a rationale for initiation of preclinical and a few clinical trials evaluating novel, isoform-specific, mutated IDH1/2 inhibitors in cancers with such genomic alteration.

Other examples of epigenetic therapy are the HDAC inhibitors: vorinostat, panobinostat, valproic acid (VPA), and entinostat. In particular, Vorinostat [67] and VPA [68] are the most tested in clinical trials on GBM as either monotherapies or combination therapies. HDAC inhibitors are known as effective therapeutic anticancer agents *via* multiple mechanisms, including the induction of cell- cycle arrest, differentiation, senescence, intrinsic and extrinsic apoptosis, mitotic

cell death, autophagy cell death, and generation of reactive oxygen species, inhibition of angiogenesis and metastasis, and improvement in tumor immunity [69]. The human genome contains 18 known HDACs, which are grouped into four classes on basis of phylogenetic analysis [70]. HDACs are overexpressed and mutated in various solid and hematologic malignancies and play key roles in tumorigenesis [71]. However, the expression and functions of HDACs in GBM are not well characterized. Recent studies have begun to focus on the expression patterns of HDACs in GBM. They are well-studied epigenetic agents that effectively radiosensitize GBM [72]. However, the exact molecular mechanism underlying HDAC inhibitor-induced radiosensitization remains elusive. Evidence suggests that it partially involves the inhibition of the DNA damage repair response [64]. Most of the GBM studies to date have focused on testing the antitumor effects of pan-HDAC inhibitors such as vorinostat and VPA rather than evaluating the role of HDAC in GBM. Despite some encouraging results from preclinical studies, early clinical trials showed only modest therapeutic benefits [67].

1.3.6 Active immunotherapy: Vaccinations (EGFRvIII-mediated vaccine; Dendritic cell vaccines)

Effective antitumor immunity in humans has been associated with the presence of T cells recognizing cancer neoantigens. The studies of adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TILs) revealed that neoantigen-specific T cells are crucial for clinical responses [73]. There are increasing neoantigen-based cancer vaccines designed to target the unique immunogenic mutations

arising in each patient's tumour. The main groups of personalized cancer vaccines the personalized RNA mutanome vaccines and peptide-based vaccines. These neoantigen cancer vaccines demonstrated to be relatively safe, feasible, and capable of eliciting strong T cell responses to neoepitopes. Treatments tailored to a person's individual cancer mutations cause the strong immune response to attack tumors.

Based on the apparent failure of glioblastoma to metastasize outside the CNS, efforts to induce active immune surveillance against glioma cells in the brain by strengthening the adaptive arm of the immune system, predominantly by vaccination, have been pursued as a promising path forward.

Three broad types of cancer vaccines are the most studied, designed in the forms of cells, proteins/peptides, and genes. However, only three vaccination approaches have reached phase III clinical development: 1) Rindopepimut (also known as CDX-110 or PEPvIII) that is a peptide vaccine that mimics and thus targets EGFR variant III (EGFRvIII); 2) IDH1 peptide vaccines; 3) dendritic cell (DC)-mediated vaccines.

In the field of GBM therapies, cell-based cancer vaccines are the most evaluated. They are autologous or allogeneic whole tumor cell vaccine and autologous dendritic cells (DC), pulsed or transfected with tumor antigens in different forms, such as tumor lysates, purified proteins, peptides, DNA, or RNA [74].

Several studies have shown the safety and feasibility of dendritic cell vaccines against glioblastoma. [75]. Despite some encouraging results, a recently published meta-analysis shows no improvements with DC therapy [76].

The authors included 3 randomized studies with a sample size of 224 patients and suggested that dendritic cell–based vaccinations have no obvious improvement in terms of median overall survival, median PFS, PFS rate, or overall survival rate as compared with control intervention for newly diagnosed GBM. On the other hand, the dendritic cell–based vaccinations are well tolerated, and no significant toxicity is reported.

However, different issues on DC vaccine are controversial. One of the most significant is the optimal timing of DC vaccinations with respect to the other treatment modalities. In the current literature, the timing of commencement of DC vaccination is by no means uniform, ranging from immediately following the surgery to several weeks after completion of radiotherapy and chemotherapy. Linked to it, there is the interaction between DC vaccinations and radiotherapy that is unclear on the involved pathways, the timing of interaction as well as the optimal doses and fractionation.

In our Institute, two clinical studies, DENDR1 (NCT04801147) and DENDR2 (NCT04002804), including respectively the treatment of first diagnosis and recurrent GBM patients with DCs loaded with autologous tumor lysate, were activated. The DENDR2 study was stopped due to lack of clear efficacy [77]; while the DENDR1 study is still active.

In this scenario, radiotherapy (RT) plays a crucial role and from the immunologic point of view, RT is thought to act as an in situ “tumor vaccine,” in that it prompts the release of tumor-associated antigens that prime an adaptive immune system.

More preclinical and clinical studies are needed to better understand how to integrate standard therapies and DC vaccines.

The main immunotherapeutic strategies under testing for GBM are summarized in Figure 4.

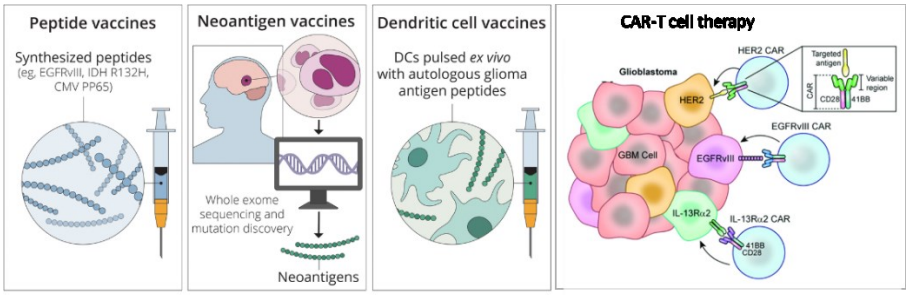

Figure 4. Different strategies of immunotherapy in experimental clinical setting (modified, Fecci et al. JNS 2019).

1.4 Radiotherapy and its immunomodulatory effects

Radiotherapy is a key cancer treatment strategy, spanning a broad range of indications from palliative to definitive intent therapy.

Ionizing radiation works mainly by inflicting double-strand breaks (DSBs), base damage and single-strand breaks (SSBs), whereas Temozolomide (TMZ), an alkylating chemotherapy agent, induces N7-methylguanine (N7- meG) and N3-methyladenine [78].

Radiation also affects organelles such as endoplasmic reticulum (ER), mitochondria, lysosomes and ribosomes. For example, radiation can cause an ER stress response leading to download from autophagic cell death or apoptosis. Radiation can also significantly affect mitochondria function through mitochondrial membrane depolarization, ROS generation and cytochrome c release, ultimately leading to apoptosis [79]. Radiation may also directly destabilize cell membrane through alteration of its composition or indirectly through ROS generation, and lipid peroxidation [79].

Until the last few years, radiation has been considered almost exclusively as a local modality. From early radiobiological studies, the major mechanism of action of radiation has been found to be mediated by DNA damage, leading to the death of irradiated cells mostly at the time of cell mitosis. However, over the past few decades, a distinct role for radiation from directly killing tumor cells has emerged. In fact, recent preclinical and clinical data suggest that radiotherapy may participate in the potentiation and modulation of tumor immunity.

In fact, in the current era of immuno-oncology, in which stimulating the immune system holds the promise of extending survival for patients with advanced cancer, radiation is taking on a new role, that of an "adjuvant" to immunotherapy.

Radiotherapy has the potential to convert immunologically 'cold' tumours into 'hot' tumors by a combination of distinct mechanisms including: (a) increasing tumor immunogenicity *via* the upregulation of antigenic expression, antigen processing, major histocompatibility molecules, and costimulatory signals; (b) overcoming an immunosuppressive tumor microenvironment by shifting the cytokine balance in favor of immunostimulation (e.g. by increasing the production of immunostimulatory cytokines); (c) recruiting antigen-presenting and immune effector cells to the tumor microenvironment (Figure 5).

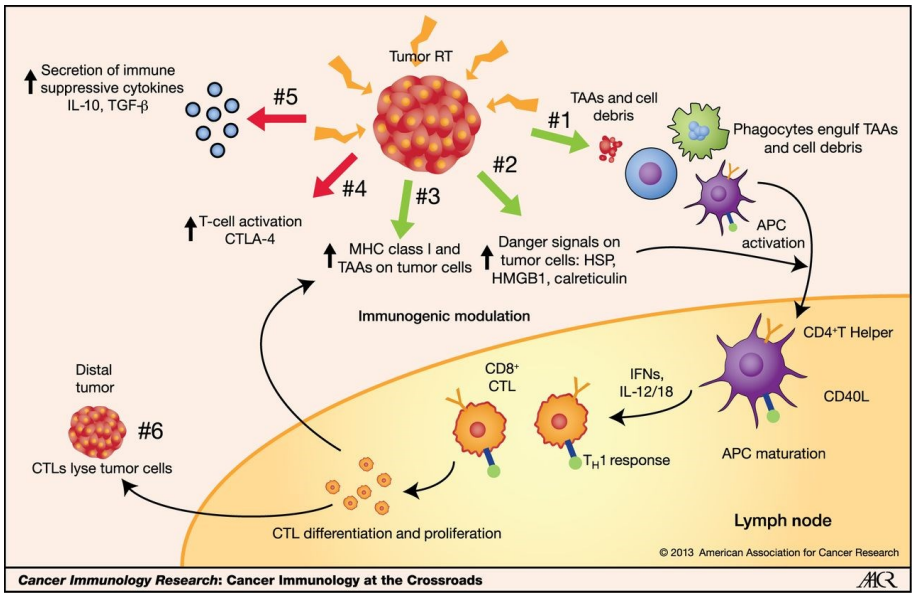

Figure 5. Immunomodulatory aspects of radiotherapy (Ahmed et al, CIR 2013.CIR-13-0141)

Moreover, tumor responses outside of the radiated field, or abscopal effects (ab scopus = outside the target), have been described, although they are rare with radiation alone [80]. For example, preclinical tumour models demonstrate that radiation exerts distant effects linked to activation of the immune system by inducing tumour-specific effector T-cells through the generation of an in-situ vaccine. [80] This mechanism may explain why combinations of radiation and immune-modulating systemic therapies have yielded higher rates of abscopal responses. In addition, in multiple preclinical studies, radiotherapy has been shown to generate tumour-specific immune responses, an effect that was lost in T cell-deficient mice or following selective depletion of CD8+ cells [80] (Figure 6).

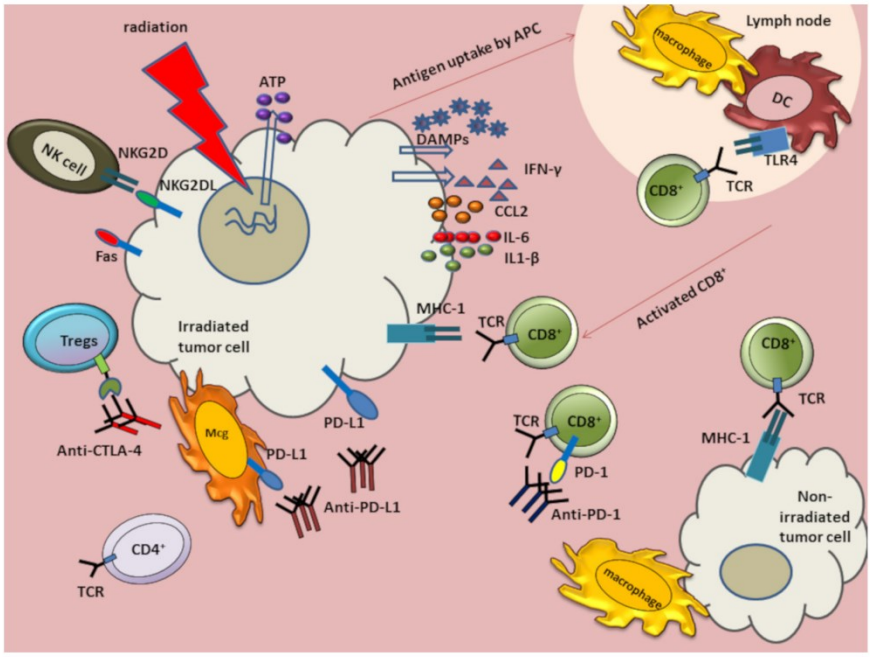

Figure 6. Abscopal effect of RT on anti-tumor immune response (Karishma R., Front Oncol . 2018

In short, radiation-induced antitumour effects can contribute to cross priming and succeed at eliciting an immune response against the tumour, besides its tumoricidal effect. However, there are many key points that are still on debate. The main uncertainties are about doses and timing of radiotherapy.

In general, RT treatment is administered over several sessions to give the normal tissue time to recover as it has better damage-repair capabilities than tumour cells. This is termed fractionation. A key problem in radiotherapy involves finding an optimal number of treatment sessions (fractions) and the corresponding dosing schedule. The idea of fractionation is directly linked to the concept of 4 R's of

radiobiology that are used to describe the cell kill process of radiation treatment [81]. Fractionated RT takes advantage of the 4 R's in the process of cellular kill: repair of sublethal DNA damage, cell repopulation, redistribution of cells in the cell cycle, and reoxygenation of previously hypoxic tumor areas. Standard radiation therapy delivers a dose of 2 Gy to the tumour at each fraction, 5 times a week during several weeks. This traditional fractionation regimen aimed at maximising the local control of the tumour while minimising the toxicity to other healthy tissues based on radiobiology models. However, such fractionation regimen might not be beneficial for all tumours, in which case the dose per fraction can be increased without lowering the quality of the treatment. This approach is called hypofractionation: the total dose of radiation is divided into large doses and treatments are given once a day or less often, given over a shorter period than standard RT. There are different levels of hypofractionation depending on the type of cancer.

In this setting, very large doses can be delivered in one to five fractions using stereotactic radiotherapy/radiosurgery, an approach that takes advantage of improvements in imaging, radiation delivery techniques, and the ability to account for organ motion in real-time. These doses usually range from 20 Gy delivered in one to three treatments to 34 Gy delivered in one treatment.

In the field of immuno-oncology, the importance of the dose and fractionation schedule has been corroborated in several studies showing activation of immune-response-related genes, radiation-induced damage-associated molecular pattern molecules (DAMPs), and inflammatory cytokines in human cancer cells when exposed to

radiation in the range of 8–10 Gy [82, 83]. Thus, these data suggest the existence of a threshold dose below which immune stimulation might be suboptimal and above which immunosuppression prevails.

Studies to optimize radiation dose and fractionation have explored a variety of altered fractionation regimens with the goal of improving the therapeutic ratio.

A single fraction of 8 Gy is probably not sufficiently immunogenic when targeting metastatic lesions, given preclinical evidence that multiple fractions may be beneficial for the abscopal effect [80]. In the PACIFIC trial, [84] patients who received concurrent chemoradiation for unresectable stage III NSCLC showed significantly improved progression-free survival (PFS) when given the anti-PD-L1 monoclonal antibody (mAb) durvalumab after chemoradiation. Although this trial did not utilize hypofractionated radiation and was in a non-metastatic population, it has been interpreted as indicating that the chemoradiation served as an immune priming event. If this is true, the addition of durvalumab was able to potentiate a systemic immune response, translating into a significant prolongation in PFS. Fractionated treatments (with radiation delivered in 2 to 3 fractions instead of a single fraction) may yield promise and reduce the risk of edema and radionecrosis. Fractionated treatments may also decrease 3' repair exonuclease 1 (TREX1) signaling and help induction of the cyclic GMP-AMP synthase stimulator of interferon genes (STING) sensing pathway. TREX1 and STING are opposing regulators of the cytosolic DNA-sensing pathway and can affect immune responses after irradiation [85] (Figure 7).

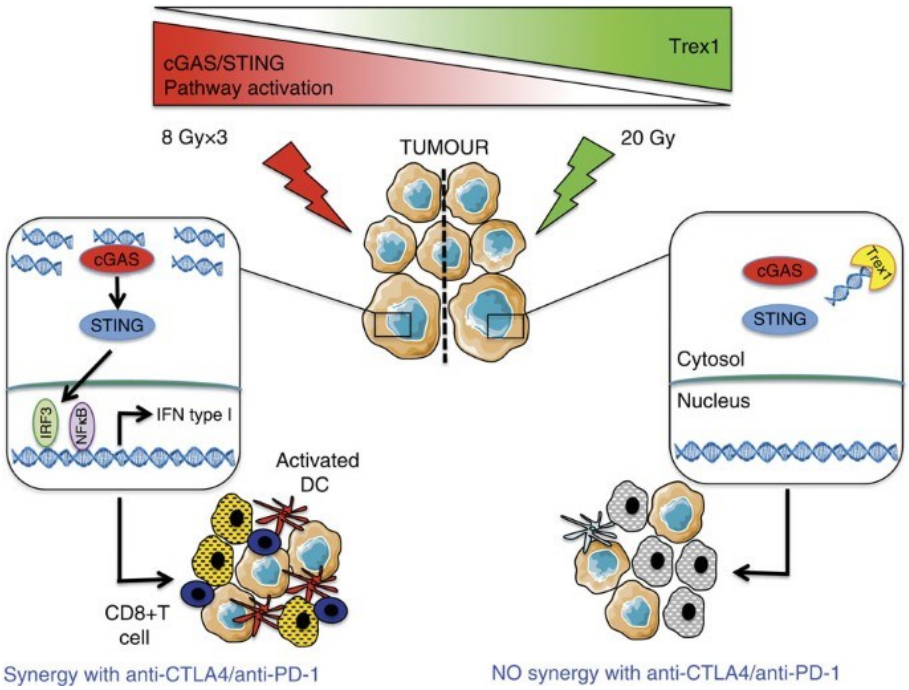

Figure 7. Role of Trex1 and the cGAS–STING pathway on DNA damage signature activated by radiotherapy (Vanpouille et al, Nature Communications 2017 vol 8:5618).

In any combination treatment that involves several treatment modalities, the timing of each component could be critical to the outcome. Since different types of immunotherapy targets, different pathways or different immune cells, the strategy of treatment combinations should be carefully designed to produce the greatest synergistic effects. To date, several preclinical and clinical studies have been carried out to interrogate this question. So far, the results appear to suggest that the optimal timing is tumor type and immunotherapy specific.

In addition, the role of RT field size play a crucial role in the immune-modulatory functions, specifically with regard to how it affects toxicity to circulating normal lymphocytes.

Overall, literature data indicate that local radiation produces systemic, immune-mediated antitumour and, potentially, antimetastatic effects [86]. Additionally, the combination of local radiotherapy and immune-modulation can augment local tumour control and cause distant (abscopal) antitumour effects through increased tumour-antigen release and antigen-presenting cell (APC) cross-presentation, improved dendritic-cell (DC) function, and enhanced T-cell priming [87] (Figure 8).

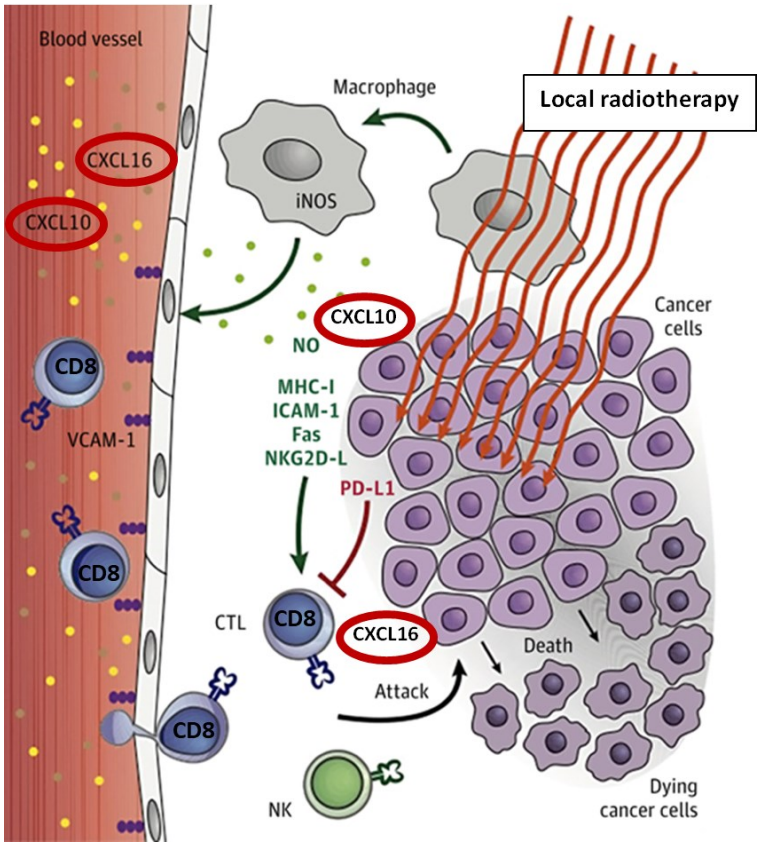

Figure 8. Local radiotherapy and effects on immune cell infiltration and homing (modified, Demaria S, JAMA Oncol.2015)

On the other hand, ionizing radiation can also generate chemotactic signals that recruit several myeloid-cell types with distinct roles in T-cell suppression [88, 89].

An increasing body of evidence, show that localized radiation initiates cell death and the production and release of cytokines and chemokines into the tumour microenvironment, which leads to infiltration of DCs, macrophages, cytotoxic T cells, and suppressive cells, such as regulatory T (Treg) cells and myeloid-derived suppressor cells, as well as the efflux of immune cells, such as DCs that are important APCs. [90].

Current evidence suggests that RT, through different pathways, can stimulate both local and systemic immune responses, which can either support tumour-cell survival or promote tumour-cell death. The balance between these effects of radiation might have a key role in determining the outcome of treatment.

1.5 Response assessment of RT-chemo and IT therapies for GBM

Standard treatment response assessment in gliomas relies on MRI. A transient increase of enhancing volume has been described up to 20-30% of patients after Stupp protocol [14–16]: this inflammatory based pseudo-progression (PsP) eventually subsides and should be distinguished from true tumor progression (TTP) to avoid early discontinuation of effective treatments. This problem is magnified with immunotherapy, which induces a stronger inflammatory response. Response Assessment for Neuro-Oncology (RANO) criteria have been proposed in 2010 [17] as a tool to address this issue with a specific

version released in 2015 (immunology RANO, iRANO) for patients enrolled in IT protocols [18]. They are based on conventional MRI (cMRI), which fails to capture the whole complexity of GBM: the size of enhancing and non-enhancing tissue is not an univocal marker of the dynamics of glioma and of immune cell interaction [19]. Moreover, with iRANO criteria, TTP can be defined only 6 months after the initiation of treatment, which is a considerable time in light of the short patient survival [18].

Advanced MRI (aMRI) techniques, including perfusion weighed imaging (PWI) and diffusion weighted imaging (DWI), can better describe tumor biology: the former describes angiogenesis and is usually elevated in malignant tissue, while the latter is an inverse marker of tissue hypercellularity (low ADC). As such they can assist in differentiating PsP from TTP and in predicting response to treatment [19–22]. Imaging evaluation during multimodal treatments and mostly IT gains specific adjunctive biases and pitfalls [18] due to the immune cells infiltrate and the CE and vessel permeability increase determined by an immune response. However, their inter- and intra-lesional variability also due to GBM nature, limits the unequivocal clinical validation of aMRI techniques in response assessment criteria.

Hence, Treatment Response Assessment Maps (TRAMs) have been proposed as a simple tool based on cMRI with potentials comparable to aMRI approaches. TRAMs require the acquisition of high-resolution 3D-T1-weigthed scans before contrast medium administration and at an early (5 minutes) and late timepoints (>1 hour) after contrast injection to determine early and late washout: they have been proposed [23] and histologically validated [24] as a tool to identify tumor tissue,

distinguishing between TTP and PsP or radiation necrosis after standard chemo-radio-therapy in high-grade gliomas and in brain metastases [25]. They have also been used to predict response to bevacizumab in recurrent high-grade gliomas [26]. To date, their application in glioma IT has never been reported in literature.

1.6 SCOPE OF THE THESIS

The main aim of this project is to sort out the immunomodulatory effects of radiotherapy for brain glioma, also in association with immunotherapy. The radiological response has been evaluated as well. This thesis summarizes the work focused on this issue.

Chapter 1. Introduction of the project and all other chapters.
Chapter 2. Radiotherapy treatment in combination with dendritic cell immunotherapy: polarization of microglia from m2 to m1 phenotype.
Chapter 3. The anti-aging klotho contributes to shape the glioblastoma immunosuppressive microenvironment. Abstract
Chapter 4. Response assessment of GBM after radio-chemotherapy and during immunotherapy by delayed contrast TRAMs (treatment response assessment maps): a pilot study.
Chapter 5. Summary, conclusions, and future perspective.

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CHAPTER 2

RADIOTHERAPY TREATMENT IN COMBINATION WITH DENDRITIC CELL IMMUNOTHERAPY: POLARIZATION OF MICROGLIA FROM M2 TO M1 PHENOTYPE

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2.1 Introduction

Radiation therapy (RT) is an integral component of oncology care, crossing a broad range of indications from palliative to definitive intent therapy. Also, for GBM patients, RT is part of the standard of care. However, despite multi-modal management, including upfront maximal safe resection followed by concurrent chemotherapy and RT and adjuvant chemotherapy, GBM has a poor prognosis. Therefore, several approaches are under evaluation to improve disease control: targeted agents, immunotherapeutic combinations, and personalized cellular vaccines. One of the most evaluated approaches is dendritic cell (DC) vaccine. Several groups [1-11] have documented proof of the principle of DC-based vaccination strategies against HGG. Two clinical studies, DENDR1 and DENDR2 (DENDR1—EUDRACT No 2008-005035-15; DENDR2—EUDRACT No 2008-005038-62) including, respectively, the treatment of first diagnosis and recurrent GBM

patients with DCs loaded with autologous tumor lysate have been being conducted in our institution. Results of many studies [12], including ours, provided evidence for feasibility and safety of DC-based GBM immunotherapy, however failed to provide convincing evidence of efficacy, raising several clinical and biological issues to be addressed in order to increase the potential of these strategies [13, 14].

DC vaccine therapy is well studied also in association with RT. As a matter of fact, several lines of investigation have provided a greater understanding that not only does RT directly influence tumour immunity, but it also exerts its effects via a series of distinct mechanisms. The main immune-modulatory effects of RT are triggering immunogenic cell death [15], generating neoantigens and enhancing antigen processing and cross presentation [16], decreasing immunosuppression in the TME [17], through reprogramming the glioma-associated microglia, overcoming T-cell exclusion from the TME, and increasing tumour cell recognition by the immune system [16].

To understand deeply the interaction between RT and DC-vaccine, we designed the present study focused on GL261-glioma bearing mice treated by means of RT and DC-vaccine.

Dissecting the TME composition and functional heterogeneity of tumor infiltrating immune cells would extend the understanding of glioma immune microenvironment and allow modulating functions of distinct subpopulations for therapeutic benefits.

2.2 Methods

All the GL261 glioma mice were randomly assigned into three groups: Model group, RT group, RT-IT group.

GL261-glioma bearing mice were locally irradiated with a total dose of 15 Gy in three consecutive fractions of 5 Gy on day 7, 8, and 9 after the tumor implantation. (Fig. 1) Irradiation was delivered both as exclusive (RT) and concomitant treatment in combination with DC immunotherapy (RT-IT). DCs were injected subcutaneously on day 16, 23, and 30 after tumor implantation. (Fig. 1 and fig. 2) Changes in the tumor microenvironment were investigated by assessing microglial and chemokine gene expression profile on gliomas of RT, RT-IT treated and unirradiated mice (controls).

To elucidate the potential role of RT in reprogramming the glioma-associated microglia, we isolated and enriched CD11b positive cells from the brain of RT, RT-IT, and control mice.

Percent animal survival were assessed for GL261-bearing mice that were either untreated or treated.

All animal studies were authorized by Institutional and national Authority.

Single-cell RNA sequencing (scRNA-seq) was used to investigate the composition and functions of glioma-associated microglia and macrophages in murine experimental GL261 gliomas grown mice.

Figures 1 and 2 summarize protocol treatment.

Figure 1. Protocol treatment

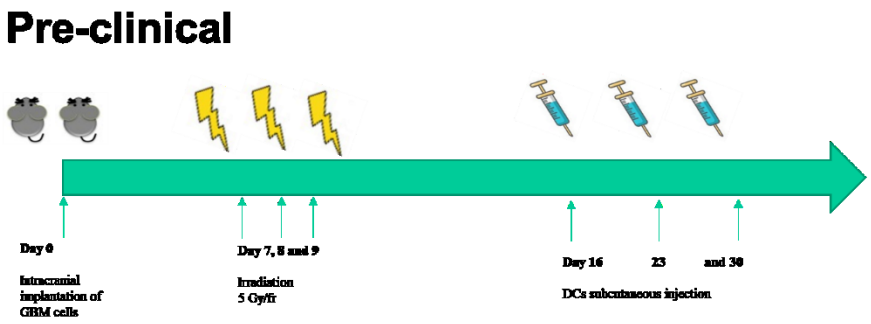

Fig.1 GL261 cells were injected in the brain of mice on day 0. RT was delivered with a total dose of 15 Gy in three consecutive fractions of 5 Gy on day 7, 8, and 9. DCs were injected subcutaneously on day 16, 23, and 30 after tumour implantation.

Figure 2. Vaccine Protocol treatment

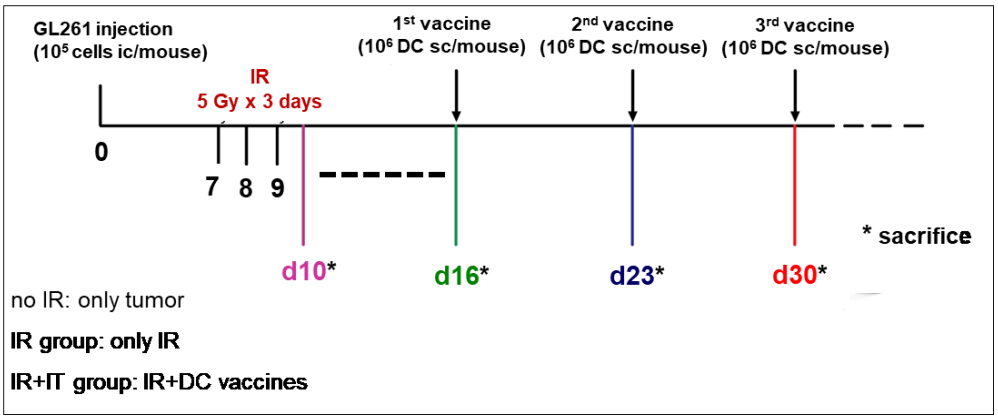

Fig. 2 DCs were injected subcutaneously on day 16, 23, and 30 after tumour implantation.

2.3 Results

Our analysis showed that RT promoted anti-tumoral M1 polarization characterized by increased production of pro-inflammatory cytokines such as TNF-α and IFN-γ and high levels of iNOS. Whereas, M2 phenotype markers, TGF-β1 and IL -10, were significantly decreased in irradiated mice on day 16.

RT induces TME modulation chemotactic gradient that facilitates the homing of immune cells to the tumor site by inducing expression and release by the cancer cells and/or infiltrating immune cells of chemokines, such as CXCL16 and CXCL10.

RT also modified chemokine expression in the TME: intratumoural expression of CCL2, CCL4, CCL5 and CXCL10 were found in irradiated mice on day 16. Moreover, RT contributes to a massive recruitment of Th1 CD4+ T cells.

In RT-IT gliomas CD45dim/CD11b+/CD172A+ activated microglia and CD4+ T cells recruitment preceded a robust infiltration of CD8+ T cells.

Survival analysis showed that glioma-bearing mice treated with the combination of RT and IT survived longer than RT mice or controls.

2.4 Discussion

Our preliminary data support the ability of RT to re-educate microglia against glioma creating an ideal inflammatory TME able to enhance DC immunotherapy efficacy. These results confirm the literature data. Preclinical models have demonstrated T cell priming after radiation [18] and increased homing of effector T cells into irradiated tumours that

display increased sensitivity to immune destruction [19]. Thus, tumour control after RT depends on T cell responses in the
host [18]. However, RT also induces changes in the TME that inhibit immune control of tumors [20].

However, there are different issues that are still controversy. Apart from the timing of DC administration, optimal RT schedule is under evaluation. Defining radiation parameters is one the most difficult aspect: number and dose of fractions, timing of delivery and size of irradiation field.

In any combination treatment that involves several treatment modalities, the timing of each component could be critical to the outcome. To date, several preclinical and clinical studies have been carried out to interrogate this question. So far, the results appear to suggest that the optimal timing is tumor type and immunotherapy-specific. Above all, in cases where the treatment modalities stimulate the functionality or stability of tumor-specific CD8+ T-cells, administration of immunotherapy following conventional therapy can improve the antitumor immune responses. For this reason, we designed our protocol applying IT after RT.

A recently published literature review on DC-vaccine therapy for GBM showed that there is a great deal of diversity in terms of patient population, timing and protocol for vaccination in the phase I/II trials investigating DC vaccine therapy. Within these limitations and potential biases, however, these trials have revealed effective stimulation of anti-glioma immune response with low toxicity.

The radiation dose and fractionation schedule are also important factors to consider when radiation is combined with immunotherapy.

Consequently, a growing body of research is active in this field, and experimental studies have already given some insight into the problem [21]

We found that RT modified chemokine expression in the TME: intratumoural expression of CCL2, CCL4, CCL5 and CXCL10 were found only in irradiated mice on day 16. In fact, several cytokines can be induced by radiation [22]. An immune-suppressive TME is triggered by reducing the cytotoxicity of CD8+ T cells, suppressing CD4+ T-cell differentiation, promoting regulatory T cell (Treg) transformation, and inhibiting natural killer (NK) cell proliferation [23]. Our results confirm these literature data.

While our understanding of the immune system's role in the response to ionizing radiation continues to evolve, novel opportunities to study how to combine immunotherapy with radiation-induced cell killing are revolutionizing cancer treatment.

2.5 Conclusion

This preclinical study aimed at defining an appropriate timing between the end of radiotherapy and the beginning of DC vaccines,

The RT fractionated protocol was selected in the attempt to handle the balance between activation of a specific anti-tumor response and the immunosuppression induced by radiotherapy.

Radiations of cancer cells trigger immunogenic cell death (ICD), which is characterized by release of damage-associated molecular patterns, also known as *alarmins*, involved in the effector cell activation and recruitment to the tumor site. These changes can also act molding the

tumor microenvironment in a pro-inflammatory antitumor immune context.

Our preclinical results confirm that RT can affect the immunosuppressive TME creating a specific chemokine gradient involved in T cell homing. RT in combination with IT can induce an anti-tumour systemic long-lasting effector CD8+ T cell response as well as a local infiltration of NK cells and CD8+ T cells.

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CHAPTER 3

THE ANTI-AGING KLOTHO CONTRIBUTES TO SHAPE THE GLIOBLASTOMA IMMUNOSUPPRESSIVE MICROENVIRONMENT

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3.1 Background

Although some progress has been made in understanding GBM, treatment remains a challenge. There is increasing evidence that radiotherapy (RT) not only provides immunomodulatory effects on tumor microenvironment (TME) but also influences systemic immune response, supporting the advantage of combinatorial strategies with immunotherapy (IT). Using immune-competent mice in which syngeneic glioma cells are grown intracranial and treated with local fractionated radiation, we assessed the effects of RT on the tumor microenvironment.

3.2 Methods

Bulk RNA-sequencing was performed on GL261 and SB28- gliomas, explanted at different time points, from mice locally irradiated with a total dose of 15 Gy in three consecutive days. Gliomas from untreated mice were used as control. Validation of selected differentially

expressed genes was performed by in vitro experiments. Changes in the tumor microenvironment were investigated by assessing infiltrating innate and adaptive immune cells.

3.3 Results

A comparison of data set derived from RNA-seq based transcriptome individuated differentially expressed genes in response to radiotherapy in GL261 glioma model; no significant differences were observed between control and irradiated tumors of SB28-glioma bearing mice. We have identified significant variation in 29 genes and among upregulated ones, we focused on klotho, known as a regulator of the interface between brain and immune system. Its expression displays significant positive correlation with GBM progression free survival. By real-time PCR we have confirmed the induction and persistence at later time points in GL261 irradiated mice compared to controls. We have confirmed dose- and time-dependent klotho over-expression in vitro in RT GL261 cells. The potential involvement of klotho in modulation of microenvironment after RT was supported by the observation of a robust infiltration of lymphocytes $CD3^+$ and a decrease of MDSC cells.

3.4 Conclusions

Our preliminary data support the correlation between klotho and radiotherapy. We hypothesize that the anti-aging klotho is released due to DNA damage and oxidative stress induced by radiotherapy and contributes to modulate tumor microenvironment.

The activation of klotho during RT, can prevent cellular senescence, acting on all the RT-related immune suppressive mechanisms.

For the future, it will be crucial to demonstrate the role of klotho in suppressing the senescent cell-associated triggering of tumor progression, and inhibiting the senescence-associated secretory phenotype (SASP), that includes a plethora of chemokines and cytokines involved in immune suppressive mechanisms.

CHAPTER 4

RESPONSE ASSESSMENT OF GBM AFTER RADIO-CHEMOTHERAPY AND DURING IMMUNOTHERAPY BY DELAYED CONTRAST TRAMS (TREATMENT RESPONSE ASSESSMENT MAPS): A PILOT STUDY.

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4.1. Introduction

Glioblastoma (GBM) is the most frequent malignant primary brain tumor. It mainly affects adults and carries a poor prognosis [1]. The current standard of care (SOC) therapy consists in maximal safe surgical resection followed by radiation therapy (RT) and adjuvant temozolomide (Stupp protocol) [2], with a median overall survival (OS) of 14-16 months. Recurrence is almost inevitable due to GBM infiltrative behavior, and there are not standard treatments for recurrent GBM. The recent successful application of immunotherapy (IT) in the care of melanoma, lung cancer and renal cancer [3] and the revolutionary discovery of a glymphatic system inside the central

nervous system (CNS) [4] have awaken the interest for IT in malignant brain tumors. Currently, more than 20% of the ongoing clinical trials in GBM are exploiting immunotherapeutic interventions. Immune checkpoint inhibitors (immunomodulators), used also for extra-cerebral tumors, have been first tested, with limited survival gain benefits [5], likely in relation to the ability of GBM to escape immunosurveillance by various mechanisms [6–10]. Vaccine-based active immunotherapies can produce a stronger immune stimulation and might overcome this limitation [11]. In particular, vaccination with dendritic cells loaded with tumor peptides has shown good safety profiles and increased OS in clinical trials [11,12]. Two clinical studies, DENDR1 (NCT04801147) and DENDR2 (NCT04002804), including respectively the treatment of first diagnosis and recurrent GBM patients with DCs loaded with autologous tumor lysate were activated at the Fond. IRCCS Istituto Neurologico C. Besta. the DENDR2 study was stopped due to lack of clear efficacy [13], while the DENDR1 study is still active. In this study, we observed that DC - immunotherapy was capable of inducing an anti-tumor immune response. The increased survival observed in responders was associated with long-lasting NK, but not CD8+ T cell response [12].

Standard treatment response assessment in gliomas relies on MRI. A transient increase of enhancing volume has been described up to 20-30% of patients after Stupp protocol [14–16]: this inflammatory based pseudo-progression (PsP) eventually subsides and should be distinguished from true tumor progression (TTP) to avoid early discontinuation of effective treatments. This problem is magnified with immunotherapy, which induces a stronger inflammatory response.

Response Assessment for Neuro-Oncology (RANO) criteria have been proposed in 2010 [17] as a tool to address this issue with a specific version released in 2015 (immunology RANO, iRANO) for patients enrolled in IT protocols [18]. They are based on conventional MRI (cMRI), which fails to capture the whole complexity of GBM: the size of enhancing and non-enhancing tissue is not an univocal marker of the dynamics of glioma and of immune cell interaction [19]. Moreover, with iRANO criteria, TTP can be defined only 6 months after the initiation of treatment, which is a considerable time in light of the short patient survival [18].

Advanced MRI (aMRI) techniques, including perfusion weighed imaging (PWI) and diffusion weighted imaging (DWI), can better describe tumor biology: the former describes angiogenesis and is usually elevated in malignant tissue, while the latter is an inverse marker of tissue hypercellularity (low ADC). As such they can assist in differentiating PsP from TTP and in predicting response to treatment [19–22]. Imaging evaluation during multimodal treatments and mostly IT gains specific adjunctive biases and pitfalls [18] due to the immune cells infiltrate and the CE and vessel permeability increase determined by an immune response.

Hence, Treatment Response Assessment Maps (TRAMs) have been proposed as a simple tool based on cMRI with potentials comparable to aMRI approaches. TRAMs require the acquisition of high-resolution 3D-T1-weigthed scans before contrast medium administration and at an early (5 minutes) and late timepoints (>1 hour) after contrast injection to determine early and late washout: they have been proposed [23] and histologically validated [24] as a tool to identify tumor tissue,

distinguishing between TTP and PsP or radiation necrosis after standard chemo-radio-therapy in high-grade gliomas and in brain metastases [25]. They have also been used to predict response to bevacizumab in recurrent high-grade gliomas [26]. To date, their application in glioma IT has never been reported in literature.

The aim of the present study is to investigate the potential of TRAMs in the definition of GBM response to dendritic cell IT plus SOC, exploring possible association with used biomarkers such as MGMt hypermethylation and to assess their diagnostic value in the distinction of PsP and TTP during immunotherapy.

4.2. Materials and Methods

4.2.1 Patient Selection

We enrolled 16 patients meeting the criteria for the DENDR1 phase II clinical trial (NCT04801147) in patients with newly diagnosed GBM. The Institutional Review Board approved the study (protocol n. 419/2014) and informed consent was obtained for all patients.

Inclusion criteria were histologically proven IDH-wt GBM, age $\geq$ 18 years and $\leq$ 70 years, residual tumor volume after surgery $\leq$ 10 cm^3 confirmed by postoperative MRI, dexamethasone daily dose $\leq$ 4 mg during the 2 days prior to leukapheresis, Karnofsky performance score (KPS) $\geq$ 70.

Sub-ependymal and multifocal diffusion of the tumor were exclusion criteria.

4.2.2 Treatment Protocol

All patients underwent surgery with subsequent leukapheresis and radiochemotherapy according to the Stupp protocol [2]. Subsequently, seven doses of vaccine were prepared according to Good Manifacturing Practices [27] and administered as described elsewhere from the same group [12]; six doses of temozolomide were also administered starting from dose 3 of the vaccine [12]. Figure 1 summarizes the schedule of treatment, as previously described [33].

4.2.3 Imaging follow-up

According to study protocol [12,21], patients underwent contrast enhanced cMRI within one week before surgery, within two days after surgery, and subsequently cMRI plus aMRI, within two days before the first vaccination, then every two months or when clinical worsening occurred. Concomitant clinical monitoring was performed according to iRANO criteria [18]. Time points are displayed in Figure 1.

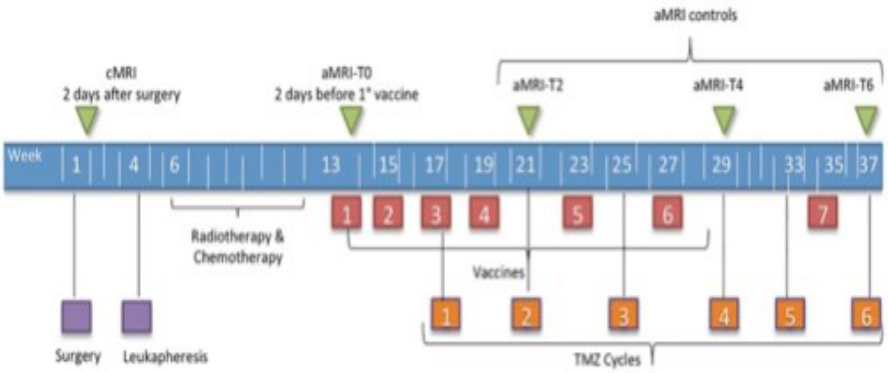

Figure 1. Timeline of the treatment regimen (below) and MRI examinations (above) of patients enrolled in the DENDR1 study protocol. Abbreviations: TMZ = temozolomide.

4.2.4 MRI acquisition

MRI was performed using a Philips 3T scanner (Achieva TX; Philips Healthcare, Best, the Netherlands) with a 32-channel head-coil.

The protocol included the following sequences: (i) 3D fluid attenuation inversion-recovery (FLAIR) (TR/TE = 4800 ms/333 ms, TI = 1650 ms, slice thickness = 1 mm, no gap, matrix = 240 x 240, Field Of View (FOV) = 240 x 240 mm); (ii) axial turbo spin-echo T2-weighted (TR/TE = 2313 ms/76.5 ms, Flip Angle (FA) = 90, slice thickness = 3 mm, matrix = 1024 x 1024, FOV = 240 x 240 mm); (iii) single-shot echo-planar DWI (TR/TE = 2936 ms/62.5 ms, slice thickness = 4 mm, matrix = 288 x 288, FOV = 288 x 288 mm, 3 orthogonal directions, b = 0–1000 s/mm2, bi-commissural acquisition) from which ADC maps were automatically reconstructed; (iv) DSC-PWI gradient-echo (GRE) (TR/TE = 1500 ms/40 ms, slice thickness = 5 mm, FA = 75, matrix = 112 x 112, FOV = 224 x 224 mm, Gadovist®,0.1 cc/Kg, 5 mL/s and fixed 3 cc pre-bolus) from which CBV maps were elaborated on a NordicICE (NordicNeuroLab AS, Norway); (v) 3D-T1 fast-field-echo (FFE) (TR/TE = 9.93 ms/4.5 ms, FA = 8, slice thickness = 1 mm, no gap, matrix = 240 x 240, FOV = 240 x 240 mm) acquired before, 5 minutes and 75 minutes after contrast-medium injection.

4.2.5 MRI post-processing

4.2.5.1 Volume Estimation

The volume of contrast enhancement (V_{CE}) of each lesion was manually segmented using MRIcro ver. 1.4 (https://people.cas.sc.edu/rorden/mricro/mricro.html#Installation).

4.2.5.2 TRAMs

TRAMs require the acquisition of two high-resolution 3D-T1 weighted scans at an early and a late time point after contrast injection. The choice of the first time point is important because right after contrast injection, the gadolinium signal rises fast, and the signal has to be high when the images are acquired in order to be sensitive to tumor regions (blue). On the other hand, this acquisition time point must be early enough not to lose sensitivity to treatment effects (red). The closer to the maximal peak value, the larger is the difference between early and delayed signal.
Both early and late post-gadolinium 3D T1 weighted scans were imported in a dedicated workstation running MatLab (https://www.mathworks.com).

The subtraction of T1-MRIs acquired 5 min post-contrast from the T1-MRIs acquired 75 minutes post-contrast yielded the color maps known as TRAMs, which represent spatial distribution of contrast accumulation/clearance. Blue color represents regions with negative subtraction values, where contrast has been cleared in late enhancement scans, as is the case of abnormal vessels proliferating within the tumor. Red color, conversely, codes for regions with positive subtraction values, where contrast stagnation happens in late scans, as is the case of inflammatory tissue.
Pre-processing of images is essential as described elsewhere, with correction of image intensity values, rigid body and elastic/local registration [23,24].

4.2.6 RANO and iRANO criteria

Definition of tumor progression is currently based on cMRI with RANO [17] and iRANO criteria [18] for standard of treatment and IT respectively. They are both based on two-dimensional measurements of enhancing and non-enhancing tissue changes. We used volumetric measurements instead of two-dimensional measurements, as recently suggested [28]. PsP was defined as an increase of enhancing tumor volume ≥40% during the first six months of IT without significant clinical worsening and with stable or regressing lesions at the following MRI without changing therapy [18,28].

4.2.7 Immune monitoring

Immune monitoring was performed on the peripheral whole blood of each patient before the treatment, after each vaccination and every two months until tumor recurrence, as described elsewhere [12].

Briefly, T-cell subsets were monitored by flow cytometry using anti-CD3-VioBlue, anti-CD4-FITC and anti-CD8-APC and anti-CD56-PE monoclonal antibodies (Miltenyi Biotec). V/B ratio for NK cell counts was used as dichotomic parameter. The ratio of the mean of vaccinations (2nd to 7th)/baseline values (V/B ratio) of NK cell absolute count for each patient was calculated, and the median of all of the observations was used as the cut-off value to separate patients into the "LOW-NK" (immunological non-responders) or "HIGH-NK" (immunological responders) groups [12]. The threshold was defined using Receiver Operating Characteristic (ROC) curves.

4.2.8. Statistical Analysis

The following TRAMs radiological parameters

were collected for each patient at different time points until tumor progression: the overall volume of contrast enhancement (V_{CE}), the volume of the red map (V_{Red}) and of the blue map (V_{Blue}); we also derived the fraction of red and blue volume over the V_{CE} (V_{Blue}/V_{CE} and V_{Red}/V_{CE}) and the percentage variation of V_{Red} (ΔV_{Red}) and V_{Blue} (ΔV_{Blue}) compared to the relative baseline evaluation (calculated as V_{Blue} / $V_{Blue\text{-}baseline}$ and V_{Red}/ $V_{Red\text{-}baseline}$ respectively).

A non-gaussian distribution of parameters was assumed. Median was used used to describe variables. Changes from baseline of radiological parameters were assessed using the Wilcoxon-Mann-Whitney tests. All p-values were two-sided. The same tests were used to determine the significance of differences in radiological parameters between different subgroups of patients (high or LOW-NK; hypermethylated and unmethylated MGMT) or between different phase of the disease (pre-progression, progression).

PFS was calculated from the first surgery until disease progression and death/last follow-up, if censored. OS was calculated from surgery to death due to any cause or last follow-up (censored). The Kaplan-Meier analysis was used to estimate PFS and OS. The log rank test assessed differences in progression or survival in patients with different radiological or clinical parameters.

Receiver operating characteristic (ROC) curves were estimated to determine for radiological parameters the value of optimal sensitivity and specificity to differentiate patients in responders or non-responders to treatment, as other biological subgroups and to distinguish TTP from PsP.

All statistical analyses were performed using SPSS 22.0 for IBM (SPSS Inc., Chicago, IL, USA) software.

4.3. Results

4.3.1 Demographics and immunological parameters.

We recruited 16 patients with histologically proven IDH-wild type GBM according to inclusion and exclusion criteria described in DENDR1 trial [12], .

Patients' main demographic and clinical data are summarized in **Table 1**.

There were 13 males and 3 females, the median age was 58 years. All patients were followed-up untill progression or death (median Follw-up 23.7 months) Fifteen cases experienced progression, only one patient died before progression because of heart failure. Seven of them had a second surgery with histopathological confirmation of recurrence tumor in 6 cases and evidence of mixed sample of tumor cells and treatment related effects in one.

Six patients had progression with the appearance of a new lesion in a region distant from the primary tumour the median PFS was 14 months. Three patients experienced PsP before evidence of TTP.

All patients were dead at the data analysis time, the median OS was 24 months. Nine patients were free from progression at 12 months. Here, we defined them as responders. PFS and OS in responder patients were statistically significant longer than in non-responder cases PFS 6 vs 16.4, p=0.0001; OS 20.4 vs 28.8 p=0.013)

Hypermethylation of the MGMT promoter, evaluated by methylation-specific PCR [29], was detected in eight of the sixteen patients.

Nine patients were defined HIGH-NK and seven as LOW-NK according to the immunological monitoring on peripheral blood. All patients with an early TTP (within 7 months of follow-up) were in the LOW-NK group. PFS and OS in HIGH-NK patients were statistically significant longer than in the LOW-NK group (PFS 6 vs 16.4, p=0.0001; OS 20.2 vs 28.8 p=0.004).

Table 1. Main demographic and clinical variables.

Patient	Age	Sex	TMZ cycles	MGMT (Met=0.1)	Vaccine doses	PFS (months)	OS (months)	2nd surgery	NK V/B ratio
1	63	M	6	M (0.21)	7	16.1	33.6	Y	HIGH
2	53	M	6	U (0.04)	7	16.4	38.4	Y	HIGH
3	50	M	6	M (2.39)	7	12.0	23.3	Y	HIGH
4†	59	F	6	U (0.00)	7	20.1	28.0	N	HIGH
5†	47	M	6	M (1.51)	7	14.0	20.2	N	LOW
6†	48	F	6	M (0.28)	7	15.1	22.6	Y	HIGH
7	55	M	6	M (1.02)	7	7.1	29.7	Y	LOW
8	68	M	1	U (0.01)	4	3.1	8.5	N	LOW
9*	58	M	6	U (0.09)	7	37.7	37.7	N	HIGH
10	50	M	2	M (1.86)	4	6.0	11.5	N	LOW
11§	65	F	3	U (0.00)	6	5.3	20.4	Y	LOW
12	58	M	6	M (0.76)	7	14.2	24.9	Y	HIGH
13	48	M	6	U (0.01)	7	16.7	23.8	N	HIGH
14	58	M	4	U (0.00)	6	5.3	15.7	N	LOW
15	69	M	6	M (0.95)	7	16.7	28.8	N	HIGH
16	65	M	6	U (0.01)	7	10.6	23.8	N	LOW

* Patient exitus before progression because of heart failure.

§ Patient with pathological evidence of mixed treatment related effects and tumor cells.

† Patients with evidence of PsP before TTP.

Abbreviations: TMZ (temozolomide); MGMT: O6-methylguanine-DNA-methyltransferase; PFS (progression free survival); OS (overall survival), V/B (vaccination/baseline).

4.3.2. TRAMs

4.3.2.1 Analyses in all patients.

A significant decline in the median of the overall volume of contrast enhancement V_{CE} and in the median of the volume of the blue map V_{Blue} was observed comparing values observed six months after immunotherapy to those detected at baseline and at 2 months (p=0.03 and p=0.013, respectively); also median of the percentage of V_{Blue} ΔV_{Blue} was significantly reduced at month 6 compared to both previous timepoints (p= 0.003 and p=0.021). The fraction of blue volume over the Vce (V_{Blue}/V_{CE}) was significantly reduced at 2 months compared to baseline.

While the median volume of the red map V_{Red} did not change at month 2 but significantly declined at month 6 compared to month 2 (p=0.033).

4.3.2.2. Analyses in TTP *and* PsP.

In the 15 patients who experienced true tumor progression and in the 3 who showed PsP during the follow-up we compared TRAMs parameters observed at progression or pseudoprogression, to those detected in the immediately previous performed exam. Due to small number of patients a formal statistical analysis of TRAMS parameters in pseudoprogression was not performed

The median V_{CE} increased both in TTP (p=0.009) and PsP.

In TTP median V_{Blu} and slightly median V_{Red} increased (p=0.007 and p=0.05, respectively) however, after normalization to baseline values, only ΔV_{Blu} showed a significant increase (p=0.013).

In PsP median V_{Blu} also increase, but the median of fraction of blue volume over the V_{CE} (V_{Blue}/V_{CE}) decreased

Using Receiver Operating Characteristic (ROC) curves a threshold variation ≥0.066 in V_{Blue}/V_{CE}) discriminated TTP and PsP with a sensitivity of 71.4% and specificity of 100% (AUC 0.875 p=0.001). Accordingly, if a variation ≥ 0.06 in V_{blu}/V_{CE} was observed the patient is predicted to have a true progression.

Figures 4, 5 and **6** display differences in TTP and PsP in two patients.

4.3.2.3. Analyses in responder and non-responder patients.

No statistically significant differences were detected between any median of the baseline TRAMs parameters detected in responder and non-responder patients. After two months of treatment the median ΔV_{Red} was significantly higher in non-responder patients compared to the responder patients, (p= 0.031).

Among responder patients, at month 6 there was a significant reduction compared to both baseline and month 2 in median V_{CE} (p= 0.015 and p=0.012 respectively), in median V_{Blue} (p= 0.008 and p= 0.017 respectively) and median ΔV_{Blue} (p= 0.008 and p= 0.021, respectively). Among non-responder patients, no significant changes in V_{CE} and TRAMs parameters were detected after two and six months Noteworthy, at month 6 only two non-responder patients were free of progression and still on follow-up.

At two months, using Receiver Operating Characteristic (ROC) curves the threshold for discriminating responder *vs* non responder patients for V_{Red}/V_{CE} variation were ≤ 0.001 with a sensitivity of 66.7% and specificity of 100% (AUC 0.754 p=0.059). Accordingly, if a reduction ≤ 0.001 in V_{Red}/V_{CE} was observed the patient is predicted to be a non-responder case.

At six months, using Receiver Operating Characteristic (ROC) curves the threshold for discriminating responder *vs* non responder patients for V_{blu}/V_{CE} variation were ≤ 0.035 with a sensitivity of 77.8% and specificity of 100% (AUC 0.88 p=0.04) Accordingly, if a reduction ≤ 0.035 in V_{Red}/V_{CE} was observed the patient is predicted to be a non-responder case. Using this threshold in dividing patients, we observed a statistically significant difference in OS (29.9 vs 23.8 p=0.009), suggesting the benefit of delayed contrast MR imaging in predicting treatment response

4.3.2.4 Analyses in HIGH-NK *and* LOW-NK patients (**Table 3**).

No statistically significant differences were detected between any median of the baseline TRAMs parameters of the HIGH- NK and LOW-NK patients. After two months of treatment the median $\Delta V_{Red\ was}$ significantly higher in HIGH-NK (n=9) patients compared to LOW-NK (n=7) patients, (p= 0.031).

Among HIGH-NK patients, at month six there was a significant reduction compared to both baseline and month 2 in median V_{CE} (p=0.015 and p=0.012 respectively), in median V_{Blue} (p= 0.008 and p= 0.021 respectively) and median ΔV_{Blue}. (p=0,05 andp=0,008, respectively).

Among LOW-NK patients, no significant changes in TRAMs parameters were detected after two and six months of treatment, apart from a mild reduction of median V_{Blue}/V_{CE} at 2 months (p= 0.043). Noteworthy, at month 6 only two LOW-NK patients had not yet undergone progression.

Using Receiver Operating Characteristic (ROC) curves the threshold for discriminating LOW *vs* HIGH-NK patients for V_{Red}/V_{CE} variation at six months was ≥ -52, with a sensitivity of 87.5% and specificity of 100% (AUC 0.875 p=0.003). Accordingly, if a reduction >52 in V_{Red}/V_{CE} was observed the patient is predicted to be a NK-high case.

No statistically significant differences were detected between any median TRAMs parameters at baseline and during treatment between patients with hypermethylated or unmethylated MGMT

Table 3. Tumor volumes (cm^3) in HIGH-NK and LOW-NK patients at baseline, month 2 and month 6.

	Baseline		2 months		6 months	
	HIGH (n=9)	LOW (n=7)	HIGH (n=9)	LOW (n=7)	HIGH (n=9)	LOW (n=2)
V_{CE}	7 (1.4 - 23.7)	12 (0.4 -51.7)	6.5 (0.1 -30.7)	7.6 (1.1 -78.8)	**2.8 (0.6-15.4)†***	6.65 (1.2 - 12.1)
V_{Blue}	3.5 (0.6-15.4)	2.4 (0.3 -27.8)	3 (0-16.7)	4.2 (0.1 -46.3)	**1.2 (0-4.7)†***	4.3 (0.3-8.3)
V_{Red}	1.5 (0.5 -11.2)	4.3 (0.1 -13.9)	2 (0-7.5)	2.5 (0.3 -24.6)	1.3 (0-3)	1.4 (0.7 -2.1)
V_{Blue}/V_{CE}	0.43 (0.28-0.87)	0.61(0.19-0.76)	0.43 (0.21- 0.6)	0.56(0.059-0.68)	0.43 (0.32 -0.75)	0.46 (0.22 - 0.69)
V_{Red}/V_{CE}	0.37 (0.09 -3)	0.27 (0.13- 0.65)	0.26(0.065-0.63)	0.32 (0.26-0.75)	0.32(0.073-0.51)	0.38 (0.17-0.76)
ΔV_{Blue}	1	1	**0.54(0.028-1.19) †**	1.52(0.048-4.62)	**0.23 (0.034-0.7)†***	0.41 (0.12 -0.7)
ΔV_{Red}	1	1	**1.52(0.18-6.26)§**	0.67 (0.031-3.6)	0.17 (0.006 -2.2)	0.29(0.09- 0.49)

§ p<0.05 compared to LOW-NK at the same time point.

† p<0.05 compared to baseline (same group).

* p<0.05 compared to month 2 (same group).

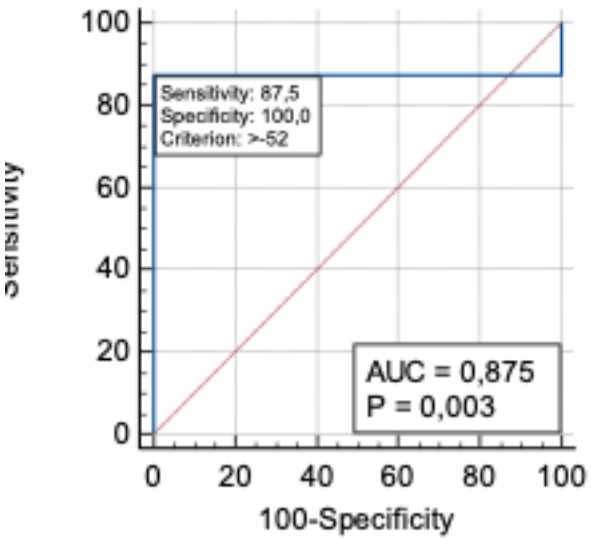

Figure 2. ROC curves assessing the ability of changes in V_{Red}/V_{CE} to discriminate HIGH-NK from LOW-NK patients.

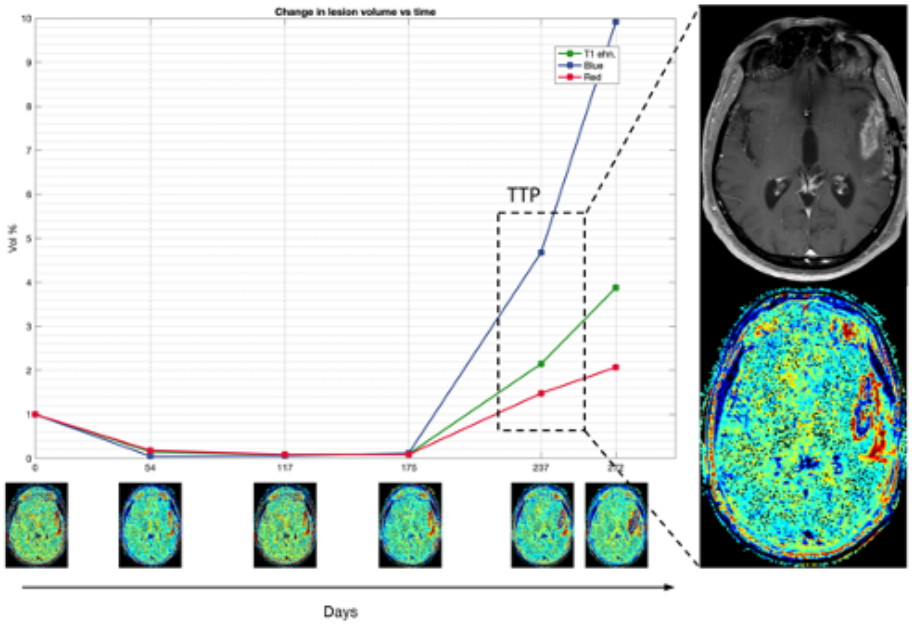

Figure 4. Patient 16 shows a classic scenario of TTP at day 237. Note the preponderant increase in blue volume between the MRI at day 175 (the pre-progression MRI) and the MRI of TTP. On the right, a

magnification of the TRAMs of day 237 is shown with the corresponding post-contrast T1 image: there is a high amount of blue color in the region of contrast enhancement.

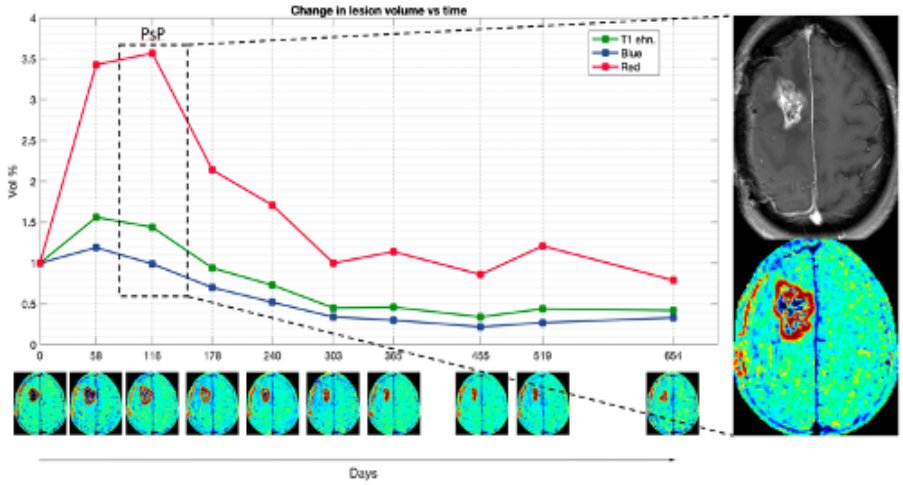

Figure 5. Patient 4 with initial suspected PsP. Note the preponderant increase in red volume in early time points, when PsP was suspected. TTP only occurs at a later time point with the appearance of a new distant lesion in the contralateral thalamus (not shown). On the right, a magnification of the TRAM at PsP is shown together with the corresponding post-contrast T1-image.

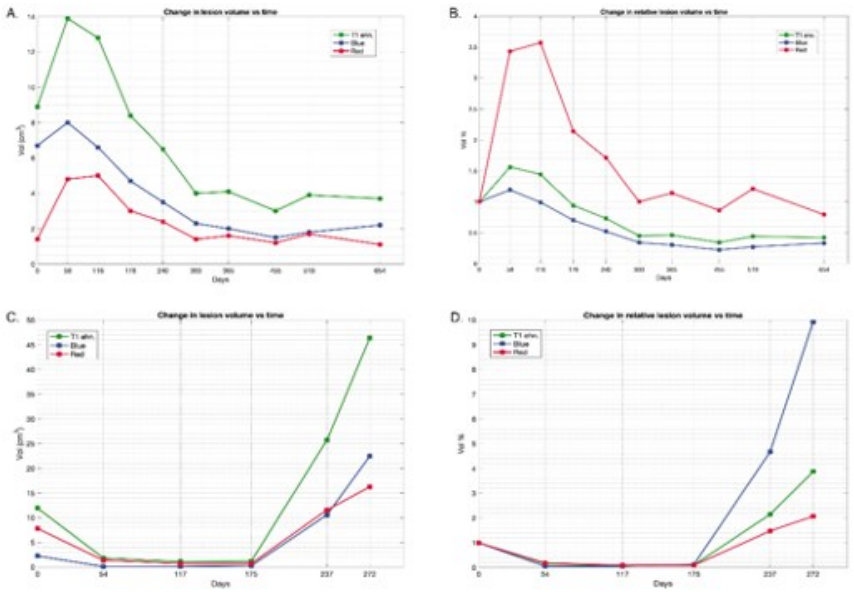

Figure 6. Comparison of absolute volumes (A, C) and relative volumes (B, D) of TRAMs in a patient with PsP (A, B panels, same patient as in Figure 5) and in a patient with classic TTP (C, D panels, same patient as in Figure 4). Note how relative volumes (ΔV_{Red} and ΔV_{Blue} respectively) better demonstrate the increase in red component at PsP (B) and of the blue component at TTP (D).

4.3.2.3. Illustrative case of a mix scenario.

Patient 11 in our cohort precociously interrupted IT due to the appearance of a new enhancing lesion in the insula showing moderate hyper-perfusion, suspect for recurrence (Figures 6 and 7).

TRAMs at the time of suspected progression mainly showed an increase in red volumes (**Figure 7**).

She underwent a new surgery with histopathological evidence of treatment related effects and rare glioma cells (**Figure 8**).

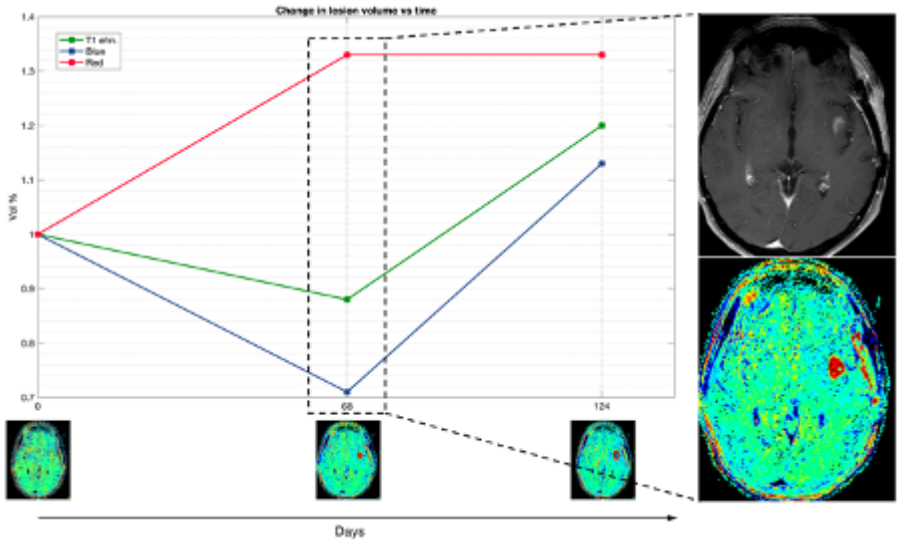

Figure 7. Patient 11 with suspected recurrence for the appearance of a newly enhancing lesion in the left insula at day 68. She underwent a new surgery with histopathological evidence of treatment related effects and glioma cells. Retrospectively TRAMs at that time point mainly showed an increase in the red volume, compatible with non-tumoral tissue: this elevation in red volume is evident both in the plot (left) and in the visual maps (right).

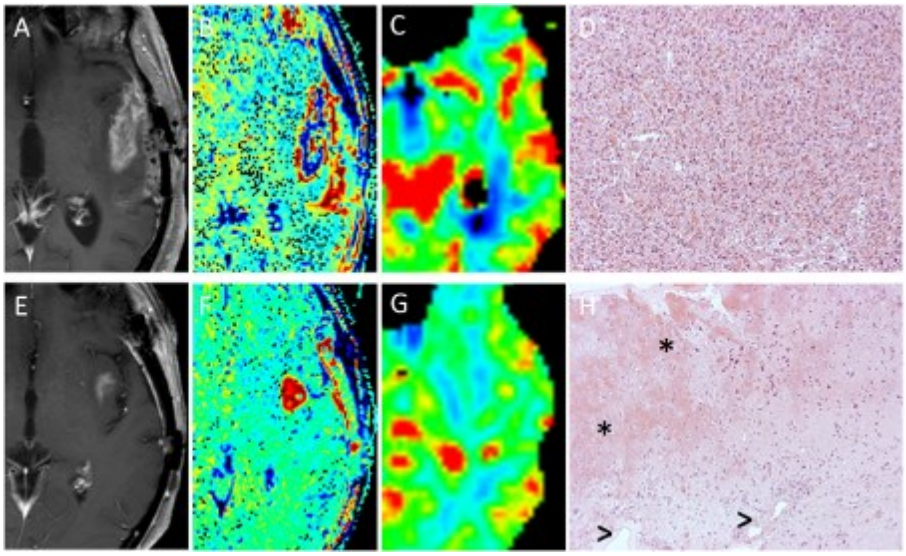

Figure 8. Comparison of a case of TTP (A-D, patient 16) and of treatment related effects with rare glial cells (E-H, patient 11). (A-D) – Pt 16: enhancing lesion (A) with elevated CBV (C), and prevalence of V_{Blue} on TRAMs (B); histopathology (D) shows a densely cellulated neoplasia with elements of marked polymorphism, frequent mitoses and presence of vascular proliferation, compatible with GBM recurrence. (E-H) – Pt 11: enhancing lesion (E) with moderately high CBV (G) and a prevalence of V_{Red} on TRAMs (F); histopathology (H) shows nervous tissue with areas of treatment-related alterations, gliosis, vessels with a thick sclero-hyalinized walls and atypical glial elements compatible with infiltration by high-grade glioma.

4.4. Discussion

GBM has a dismal prognosis in most cases, besides multimodal standardized and experimental treatments.

It is a quite novel treatment modality for GBM, known to be an immune-suppressive tumor, to induce inflammatory response within

the tumor environment. To date, SOC for GBM treatment approach relies on RT and TMZ chemotherapy according to Stupp protocol, thus it can be added to SOC but cannot replace it.

Confident and early identification of TTP is vital to avoid continuation of non-effective therapies and possibly to switch to an alternative treatment regimen. Standard accepted response assessment criteria (iRANO), based on cMRI, are not able to univocally discriminate between TTP and PsP, which is a non-tumoral radiological expression of treatment-related tissue inflammatory alteration. Moreover, there is no validated MRI surrogate marker of immunological response, even if changes in CBV, k^{trans} and ADC have been proposed as possible ones [19,21].

TRAMs are based on cMRI T1-contrast enhanced volumetric sequences and can be easily performed on ≥1.5 Tesla scanners. The technique exploit the principle of delayed contrast imaging as the subtraction of late and early post-contrast scans allows the identification of areas of early contrast clearance (conventionally blue colored, hypothesized to be tumoral, due to neoangiogenic vessels) and of contrast accumulation (red, hypothesized as treatment-related). Blue volumes have been histologically validated in patients receiving radiotherapy as a surrogate marker of tumor tissue, while red volumes have been demonstrated to be non-tumoral tissue [24].

TRAMs have never been reported in the literature as a possible tool to address these issues in patients undergoing IT. In our pilot study we applied TRAMs in a homogeneous cohort of 16 patients with GBM IDH-wt treated with surgery followed dendritic cell-based IT added to SOC.

In TTP median V_{Blu} and slightly median V_{Red} increased (P=0.007 and P=0.05, respectively) however, after normalization to baseline values, only ΔV_{Blu} showed a significant increase (p=0.013).
In PsP median V_{Blu} also increase, but the median of fraction of blue volume over the V_{CE} (V_{Blue}/V_{CE}) decreased and using ROC curves a threshold variation ≥0.066 in V_{Blue}/V_{CE}) was able to discriminate TTP and PsP

An increase of V_{Blu} would be expected in cases of TTP since the blue region, that is hypothesized to correspond to tumor tissue, is the one that should rise most significantly upon progression. However, our data suggest that instead of raw data, the entity of the variation of the fraction of V_{Blu} over the V_{CE} should be considered
Biases and pitfalls in radiological assessment of response during immunotherapy are peculiar and add challenges to multimodal treatment mix scenarios and to GBM that is an un-homogeneous tumor on its own [17].
Other advanced MRI techniques have already been studied as a possible early marker of progression in the setting of GBM and IT: on PWI, elevated CBV values within a region of contrast enhancement have been shown to support a diagnosis of tumor progression [20,30], while reduced CBV in the context of an enhancing lesion in GBM has been proposed as a possible marker of PsP [31,32]. However, evaluation of CBV and other PWI parameters is limited by the location of the lesion, as cortical lesions suffer from the physiological high perfusion of the cortex that might mask tumor perfusion; moreover, PWI has a quite low spatial resolution and might miss small lesions. On the other hand, PWI-

DCE-K^{trans} overtakes tumor location but is related to tumor vessel permeability that may also be impaired by loosening of endothelial tight junctions due to inflammation in IT. Finally, no clear univocal cutoff for aMRI values has been defined to differentiate TTP from PsP, which often overlap.

The TRAMs are simple to acquire, as they only need high quality 3D-T1 imaging, which have high resolution and do not have artifacts near the cortex nor suffer from susceptibility phenomena. Moreover, they are potentially easier to interpret since late enhancement is either present or absent. The main issues of the technique are the determination of the soil to discriminate red *vs* blue tissue to obtain adequate maps; the validation of the "red tissue" (hypothesized to be present in PsP or in mixed scenarios) on surgical specimens, because second surgery in GBM is usually performed only in selected cases and when TTP is strongly suspected. All the six patients who underwent second surgery due to suspect TTP and had prevalence of V_{Blue} on TRAMs gained histological diagnosis of recurrence. Nevertheless, the presence of residual both blue and red volume in many patients in our cohort indicate that probably tumor cells and inflammatory infiltrates coexist in the same patient, as highlighted by the case we anecdotally displayed with histological evidence of both treatment-related effects and minority of tumor cells (patient 11, Figure 7 and 8) and prevalent V_{Red} on TRAMs before second surgery.

The TRAMS could also provide additional information regarding cases responsive to IT. Only in responder patients, at month 6, we observed a significant decreased, in median V_{Blue} ($p= 0.008$ and $p= 0.017$ respectively) and median ΔV_{Blue} ($p= 0.008$ and $p= 0.021$ respectively

compared to both baseline and month 2 median values. Furthermore, at the same timepoint, a threshold ≤ 0.035 for V_{blu}/V_{CE} variation was able to discriminate responder *vs* non-responder cases. The value of this finding was also conformed using log-rank showing statistically significant differences in OS. Detecting the radiological and immunological characteristics of responder cases will provide precious information for guiding the optimization of future treatments

In a previous study from our group, dendritic cell vaccination induced a significant, persistent activation of NK cells associated with prolonged survival [12]. We therefore stratified our patients in HIGH-NK and LOW-NK as previously described [12]. No difference in CE or TRAMs at baseline was detected between LOW-NK and HIGH-NK patients. All patients with early progressive disease (<7 months) in our cohort belonged to the LOW-NK category and had a shorter OS, too. HIGH-NK patients had a significant reduction in V_{CE}, in V_{Blue} and in ΔV_{Blue} at month 6 compared to both previous time-points, and at month 2 compared to baseline interpretable as a reduction in tumor volume and, therefore, as an indirect sign of tumor response to IT in HIGH-NK patients as opposed to LOW-NK patients, who mostly undergo early progression.

Moreover, only HIGH-NK patients had a trend to increase in red volumes at month 2 compared to baseline: we hypothesize it could represent an initial increase in non-tumoral enhancement due to an inflammatory response with immune cells infiltrate. HIGH-NK patients do indeed have a better response to therapy than LOW-NK patients, as witnessed by their longer PFS and OS. In a previous work on a larger cohort of patients including the ones of the present study, we reported

that after the 4th dose of vaccine (i.e. 2 months from the beginning of IT) a reduction in minimum rADC values was visible only in HIGH-NK patients and not in LOW-NK patients. We attributed this phenomenon to an increased cellularity in the affected tissue due to an immune infiltrate. We therefore now hypothesize that the apparent increase in red volumes at month 2 in TRAMs and the concomitant reduction in minimum rADC might be different features of the same cellular infiltrate that is at the basis of immune response in HIGH-NK patients as opposed to LOW-NK patients. The ROC curve analysis confirms that changes in the V_{Red}/V_{CE} correlate with an elevated peripheral blood NK cell count, as a possible marker of immune response.

Identification of early imaging markers of tumor response is at least as important as the early discrimination between TTP and PsP: it could help clinicians to better tailor therapies and could have potential role in the assessment of tumor response in clinical trials. Most of MRI studies have focused on the distinction between TTP and PsP rather than on the identification of markers of tumor response. Reduced ADC values, which have been proposed as a marker of immune cell infiltration and tumor response [21], could also be detected in case of progressive disease due to tumor cellularity [20]; concomitant evaluation of ADC and of CBV can assist in defining the correct scenario. Our pilot study demonstrates that TRAMs might be a potential alternative or an additional tool in the distinction between TTP and PsP, and they might provide early markers of tumor response.

4.5. Conclusions

Our considerations are derived from a pilot study with patients on experimental treatments and are therefore intrinsically limited by the low sample size. The statistical power of our analyses is therefore limited. It is therefore important to further study TRAMs in larger cohorts of patients on similar treatments, ideally with the concomitant use of additional aMRI to compare the diagnostic and prognostic values of different imaging techniques, and with histological validation when feasible. The present study is preliminary to the RF study "Radiomics, circulating biomarkers and transcriptomics to dissect immune responses to radiotherapy and immunotherapy of glioblastoma" approved by the Italian Ministry of Health (RF-2019-12371008; PI MG Bruzzone, Co-PI V Cuccarini).

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CHAPTER 5

5.1 Summary

Glioblastoma (GBM) is a fast-growing and aggressive brain tumour. GBM is the most frequent malignant primary brain tumour and it can result in death in three-six months, if untreated. The current standard of care therapy consists in maximal safe surgical resection followed by radiation therapy and adjuvant temozolomide (Stupp protocol), with a median overall survival (OS) of 8-10 months. However, more than half of GBM patients die within one year from the diagnosis, and only 5% survive more than 5 years despite aggressive therapies. Research has now shifted additional attention to methods of modulating the innate immune system for the treatment of GBM. Moreover, radiotherapy, that plays a key role in GBM treatment, has

the potential to convert immunologically ‘cold’ tumors into ‘hot’ tumors by a combination of distinct mechanisms. Overall, literature data indicate that local radiation produces systemic, immune-mediated antitumour and, potentially, antimetastatic effects. Additionally, the combination of local radiotherapy and immune-modulation can augment local tumour control and cause distant (abscopal) antitumour effects through increased tumour-antigen release and antigen-presenting cell (APC) cross-presentation, improved dendritic-cell (DC) function, and enhanced T-cell priming.

The scheduling of RT and IT has been suggested as an important issue for synergizing RT and IT. In order to sort out the immunomodulatory effects of radiotherapy for brain glioma we conducted this project, also in association with immunotherapy by means of DCs loaded with autologous tumor. The radiological response has been evaluated as well. GL261-glioma bearing immune-competent mice were treated by means of RT (3 fractions, 1 fr/day) as exclusive and concomitant immunotherapy (DC), and local and peripheral modulation of the immune response were evaluated showing that RT can exert an important immunomodulatory effect on the TME, affecting the immune suppressive component and favoring the recruitment of effector T cells.

In particular, RT promoted antitumoral M1 polarization and contributed to a TME modulation, that promoted a massive recruitment of *Th1* CD4+ T cells. Notably RT and DC combination contributed to a robust infiltrate of CD8+ T cell within the TME and a long-lasting increase of peripheral CD8+ T cells.

Although preliminary, our data can be useful to identify a potentially optimal time-frame for administering IT during or after RT in GBM patients.
In addition, a comparison of data set derived from RNA-seq based transcriptome individuated differentially expressed genes in response to radiotherapy in GL261 glioma model. Significant variation in 29 genes were found, and among upregulated ones, klotho, known as a regulator of the interface between brain and immune system, displays significant positive correlation with GBM progression free survival. The induction and persistence at later time points in GL261 irradiated mice compared to controls was found. Moreover, dose- and time-dependent klotho over-expression in vitro in RT GL261 cells has been confirmed by our study. In this context, klotho could be a crucial TMEmodulator, with a specific role in shaping the immunosuppressive myeloid compartment.
The information gained during RT may help to find a time window for DC administration during and not after RT.

Exploiting the ongoing clinical study DENDR1, we have triedto explore the RT immune-effects on GBM patients treated with standard therapy plus DC-vaccine therapy DC compared with patients treated with the standard of care (SOC) only.
Response assessment of GBM after radio-chemotherapy and during immunotherapy by delayed contrast TRAMs (treatment response assessment maps) was evaluated as well, considering that the accuracy of TRAM per se or in combination with advanced MRI may be

instrumental to define progression in the follow-up of GBM patients undergoing DC IT + SOC.
In vitro and *in vivo* studies are still ongoing with the aim of more deeply examining the correlation between RT doses and immune-effects as well as the optimal timing and schedule of concomitant RT and immune-therapy.

5.2 Conclusions

Our results confirm that RT can modulate the TME creating a specific chemokine gradient involved in T cell homing. RT in combination with IT can induce an anti-tumour systemic long-lasting effector CD8+ T cell response as well as a local infiltration of NK cells and CD8+ T cells. The combinatorial approach seems to be a promising therapy for GBM patients. It might be evaluated trough other clinical trials in order to confirm the preliminary results.

5.3 Future Perspectives

So far, we have learned that the GBM microenvironment lacks cytotoxic T cells and contains abundant immunosuppressive macrophages and myeloid-derived suppressor cells.
Future trials could aim to overcome the immunosuppressive GBM microenvironment via approaches that address lack of T cell infiltration (oncolytic viral therapies, vaccine peptides, dendritic cell vaccines, and CAR T cells), lack of success with antigen selection in GBM (NKcells), T cell activation (antibodies against T cell stimulatory ligands and pro-inflammatory cytokines), and maintenance of T cell activation (TGF-β inhibition).

Radiotherapy plays a key role as both direct treatment and immunomodulator. More deep understanding of the related mechanisms and the effects on immune system of radiation should be addressed by future studies to design the optimal tailored therapy for GBM patients.

We are working on the identification of specific immunological markers in the periphery, that may reflect the modulation of the GBM microenvironment by RT and DC IT +SOC and be supportive of MRI findings. Based on the evidence that KLOTHO can be released and found in the serum of patients, we are also considering the possibility to monitor this factor performing ELISA assays on the serum of patients at different time points of DC IT +SOC compared with SOC. Changes in KLOTHO levels might reveal alterations in the immune contexture composition and might be useful to assess the optimal scheduling of RT and IT.

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