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FACULTEIT GENEESKUNDE
BIOMEDISCHE WETENSCHAPPEN

Veranderingen in de immunobiologie van maligne hersentumoren door radiotherapie, chemotherapie en immuuntherapie

Changes in the immune microenvironment of malignant brain tumors after radiotherapy, chemotherapy and immunotherapy

Masterproef voorgedragen tot het behalen van de graad van Master in de biomedische wetenschappen door

Roxanne WOUTERS

Promotor: dr. An COOSEMANS
Copromotor: dr. Matteo RIVA
Laboratory of Tumor Immunology and Immunotherapy

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VOORWOORD

Beste collega's, familie en vrienden,

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ARG-1	Arginase 1
BBB	Blood-brain barrier
B_{reg}	Regulatory B cell
CBTRUS	Central Brain Tumor Registry of the United States
CNS	Central nervous system
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMP	Danger associated molecular pattern
DC	Dendritic cell
EGFR	Epidermal growth factor receptor
EMA	European Medicine Agency
EORTC	European Organization for Research and Treatment of Cancer
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
GBM	Glioblastoma
GM-CSF	Granulocyte macrophage colony-stimulating factor
gMDSC	Granulocyt-like myeloid derived suppressor cell
HGG	High grade glioma
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukine
LAK	Lymphocyte activated killer cell
LGG	Low grade glioma
Ly6C	Lymphocyte antigen 6 complex locus C
Ly6G	Lymphocyte antigen 6 complex locus G
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MDSC	Myeloid-derived suppressor cell
MF	Macrophage
MGMT	Methylguanine-DNA methyltransferase
mMDSC	Monocyte-like myeloid derived suppressor cell

MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	National Institute of Health
NK	Natural killer cell
NKG2D	Natural killer group 2D
NO	Nitric oxide
NOS	Not otherwise specified
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death receptor 1
PDGFR	Platelet derived growth factor receptor
PD-L1	Programmed cell death ligand 1
PI3K	Phosphoinositide 3 kinase
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
TAM	Tumor associated macrophage
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TGF	Transforming growth factor
TIL	Tumor infintrating lymphocyte
TIL-B	Tumor infiltrating B cells
TLR	Toll like receptor
TMZ	Temozolomide
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

ABSTRACT

Glioblastoma (GBM) patients have a median survival of only 14,6 months despite aggressive standard treatment (surgical resection followed by radio-chemotherapy). So far, combination of immunotherapy and conventional therapies failed to improve outcome. The aim of this study is analyze the local immune-modulatory effects of radiotherapy, chemotherapy and immunotherapy and to provide evidence to improve the current therapeutic combinations.

An orthotopic CT-2A glioma model was generated in C57BL/6J mice. Treatments consisted of radiotherapy (RT, 4 Gy), Temozolomide (TMZ, 4 x 50 mg/kg) and anti-PD-1 (3 x 100 µg) used as single and combination treatments. Readouts included survival, variation in tumor volume (magnetic resonance imaging) and analysis of tumor-infiltrating immune cells (flow-cytometry).

All treatments had measurable effects on the tumor microenvironment. As single agents, RT had the strongest immune-stimulatory and TMZ the strongest immune-inhibitory effects, while anti-PD-1 had only limited effects. This was reflected in survival curves. In RT – TMZ treated mice (clinical standard of care) the survival benefit (compared to RT) alone was due to its direct cytotoxic effects since it reduced immune activation. Anti-PD-1 was not sufficient to counteract the detrimental effects of TMZ. No RT – TMZ – anti-PD-1 combination significantly prolonged survival compared to RT – TMZ.

Our results indicate the importance of the immune-modulating effects of treatments on the immune microenvironment. The inefficacy of anti-PD-1 to revert the immune suppressive activity of TMZ may explain its failure in clinical trials so far performed and highlights the need for more synergistic and effective combination strategies.

1. Introduction

1.1. High grade gliomas: the great enemy

1.1.1. Classification

The term “glioma” is used to describe a group of neuroepithelial tumors arising from the supportive tissue of the central nervous system (CNS), namely the glial cells. Gliomas can arise anywhere in the CNS, but are most often located in the brain. A relatively simple histologically-based classification can be made based on the supposed cell of origin of the tumor. Astrocytomas, oligodendrogliomas and ependymomas arise from astrocytes, oligodendrocytes and ependymal cells respectively. A mixed tumor type (oligoastrocytic tumor) is defined as well. This classification is mainly based on light microscopic characterization in hematoxylin and eosin-stained sections, immunohistochemical protein expression and ultrastructural characterization, and does not take into account the genetic differences (1,2).

Gliomas can also be divided into subcategories depending on their grade of malignancy as described by the World Health Organization (WHO) in 2007 (3). This classification is mainly based on histopathological features, such as infiltration capacity, mitotic activity and the extent of necrosis. Low grade gliomas (LGG) include WHO grade I and II and are characterized by a less malignant phenotype. The WHO grade III applies to tumors that show clear histological evidence of malignancy such as nuclear atypia and mitotic activity (anaplastic astrocytomas, anaplastic oligodendrogliomas and anaplastic oligoastrocytomas). Lastly, grade IV tumors are defined by the presence of necrosis and microvascular proliferation and are typically associated with a fatal outcome. Among these grade IV gliomas are the glioblastomas (GBM), astrocytomas with the highest grade of malignancy. WHO grade III and IV tumors are classified as high grade gliomas (HGG) (3–5). The 2007 WHO classification system was updated in 2016 in order to incorporate the characterization of the genetic background of the tumors and the presence of specific alterations in molecular pathways (2). With this new classification system, some new entities were included and other entities disappeared. An important addition in the GBM classification, is the distinction between isocitrate dehydrogenase gene (IDH) wildtype, IDH mutant and not otherwise specified (NOS) GBMs. This distinction can also be applied to oligodendrogliomas. Furthermore, a new

variant of GBM was added to the classification, namely the epitheloid GBM which joins giant cell GBM and gliosarcoma both under the IDH-wildtype GBM. Lastly, the diagnosis of an oligoastrocytoma is strongly discouraged in this 2016 CNS WHO classification. It should be either classified as astrocytoma or oligodendroglioma with genetic testing (2).

1.1.2. Epidemiology and prognosis

Glioblastoma is the most common form of primary malignant tumor of the CNS. It accounts for 45,2% of the malignant primary brain and CNS tumors and for 54% of all gliomas in the United States based on results of the Central Brain Tumor Registry of the United States (CBTRUS) (6). In Europe, the age-standardized incidence rate for astrocytic gliomas, including GBM, was calculated at 4,4 per 100.000 using data obtained from different national cancer registries in the period between 1995 and 2002 (7). Malignant gliomas and in particular GBM appear to be more common in the male population and in Caucasian population (6–8). According to the previsions of the Belgian Cancer Registry, new diagnoses of malignant CNS tumors will increase of 8,6% in 2025 compared to the year 2014 (9).

Glioblastoma is a disease with very high mortality and morbidity. The currently available standard treatment can prolong the life of GBM patients, but is still not able to provide an actual cure (8). The prognosis for GBM patients is described in months rather than years, indicating the very poor prognosis associated with the disease (10,11). Since concomitant and adjuvant Temozolomide (TMZ) was added to the standard treatment schedule, which initially solely consisted of surgical resection combined with radiotherapy (RT), the median overall survival has slightly increased from 12,1 to 14,6 months (12). Nevertheless, no significant improvements of the overall survival were seen in the following ten years (11), indicating the need for a better treatment strategy to be offered to these patients. The Belgian Cancer Registry (2004-2008) confirms that prognosis associated with high grade astrocytic tumors is very poor: the five year relative survival is 12,3% in males and 16,2% in females. The median survival of GBM patients is only 12 to 15 months when receiving the standard treatment, based on a combination of surgical resection, radiation and chemotherapy (12,13).

1.1.3. Symptoms and diagnosis

General symptoms of HGG patients are explained by the increased intracranial pressure due to tumor growth and consist in headache, nausea and vomiting. Depending on the functional aspects of the affected brain area, specific symptoms can also be present, such as walking imbalance, cognition and personality changes, urinary incontinence, memory disorder, alteration of language, loss of vision and others (14–16). Seizures are not uncommon in HGG patients, even if they are more frequent in LGG (14). However, in most cases the symptoms are unspecific; therefore a correct diagnosis solely based on clinical aspects is usually not possible (17).

At present, magnetic resonance imaging (MRI) has become the primary diagnostic tool for CNS tumors, in particular for HGGs, whereas computed tomography (CT) is usually performed only for cases in which MRI is not feasible (16,17). Different types of MRI sequences can be used. The traditional T1 and T2 weighted images are used to distinguish the tumor tissue from the normal brain based on morphology, while contrast enhancement with gadolinium can give more information about the vascularization, tumor cell density, necrosis and the degree of disruption of the blood-brain barrier (BBB) (18–20). Magnetic resonance spectroscopy (MRS) is another tool that became important in the diagnosis of GBM. Using this technique, the presence of different metabolites within the tumor can be detected. This non-invasive technique plays an important role in differential diagnosis, helping to distinguish between neoplastic and non-neoplastic lesions, before even doing a biopsy (20–22). At present, great technological advances have made available several MRI techniques which improved our non-invasive diagnostic capacity. However, despite the high degree of accuracy that these techniques can offer, diagnostic certainty can be obtained only with histopathological examination after tumor resection or biopsy (17,20).

1.1.4. Molecular heterogeneity

The Cancer Genome Atlas (TCGA) initiative launched by the National Institute of Health (NIH) played a fundamental role in unraveling the molecular heterogeneity of GBM (23). The purpose of the TCGA is to shed light over the genomic alterations present in cancers, in order to create an “atlas” of genomic profiles for over 30 human tumor types. By means of high throughput genomic sequencing and integrated bioinformatics data analyses, researchers provided wide

datasets and made them accessible to everyone for analysis. The main goal is to help improve diagnostic possibilities, treatment options and ultimately try to prevent cancer in the future (24). A combination of genomic profiling (25) and the TCGA initiative (23) identified three main pathological molecular mechanisms commonly showing an abnormal function in GBM, involving tumor suppressor protein p53, retinoblastoma protein (RB) and the receptor tyrosine kinase/rat sarcoma/phosphoinositide 3 kinase (RTK/Ras/PI3K) pathway. Alterations in these molecular systems allow malignant cells to escape from the cell cycle checkpoints, avoiding senescence and apoptosis (26–28).

Growth factor signaling pathways can be significantly deregulated in GBM. These defects often involve epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR). EGFR amplification occurs almost exclusively in primary GBM. The PDGF signaling is an important regulator in the development of glial cells. Ligands and receptor of this pathway are often expressed in gliomas and generate a stimulatory proliferative signal for the tumor. Activation of both growth factor receptors results in stimulation of cell survival, proliferation, invasion and angiogenesis through multiple signaling pathways, such as the Ras – mitogen activated protein kinase (MAPK) pathway and the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway. Phosphatase and tensin homolog (PTEN), a tumor suppressor protein, is inhibited by the activation of the PI3K pathway and has been found to be inactivated in GBM patients. As an effect of the alteration of these pathways, also vascular endothelial growth factor receptor (VEGFR) can be upregulated in GBM, ultimately leading to uncontrolled angiogenesis (8).

Based on biological and genetic differences, an important subdivision can be made: primary and secondary GBM (8). The first arise *de novo* while the latter arise by definition from diffuse astrocytomas (WHO grade II) or anaplastic astrocytomas (WHO grade III) (29). On a genetic basis these two types of GBMs show relevant differences. For example, mutations of p53 are very rare in primary GBM but are commonly found in secondary GBM. The opposite happens for EGFR overexpression (25). This indicates that these two types of tumors arise from the alteration of distinct molecular pathways. In general the genetic alterations that are associated with primary GBM include EGFR amplification, PTEN mutation and the loss of chromosome 10. In secondary GBM, p53 mutations and chromosome 19q loss are more commonly present (25).

In term of impact on patients' survival, the most important genetic alterations are the mutations of IDH and the methylation status of the O^6-methylguanine-DNA methyltransferase (MGMT) promotor (30). The isoenzyme IDH1 is an important catalytic enzyme involved in the oxidative carboxylation of isocitrate to α-ketoglutarate, resulting in the production of nicotinamide adenine dinucleotide phosphate (NADPH). Mutations in IDH1 isotype in gliomas are located in the active site of the enzyme that is critical for isocitrate binding, inactivating the protein's ability to carry out its catalytic activity. The result is a reduced level of NADPH, an important cofactor that is necessary to provide sufficient levels of glutathione, essential in the control of oxidative damage (25,31). Genetic alterations in the IDH genes have been associated with a significant increase in overall survival. Patients with mutations in IDH1 had a median overall survival of 3,8 years compared to 1,1 years for patients with wildtype IDH1 (25), indicating the clinical significance of IDH1 mutations in the prognosis of GBM patients (32). The methylation status of the MGMT promotor is another important factor when considering the molecular heterogeneity of GBM and its prognosis. This MGMT status is especially important when treating patients with alkylating chemotherapeutic agents such as TMZ (a drug that is used as part of the standard treatment for GBM) (12,13). It kills cancer cells by adding an alkyl group to the O^6 position of guanine and damaging the DNA. MGMT removes this alkyl groups from this position; therefore, high MGMT expression in cancer cells is associated with resistance to alkylating agents. Via methylation of the CpG islands in the MGMT promotor a form of epigenetic gene silencing is put in place and the MGMT production is decreased, increasing the sensitivity to alkylating agents and improving prognosis (33,34).

1.1.5. Risk factors

Many epidemiological studies have tried to identify environmental risk factors or carcinogens that are related to glioma development. For instance, some job occupations (farmers, physicians and firefighters) have been linked to an increased glioma risk in some studies (35–37). The increased risk for farmers can be linked to their exposure to pesticides (38). Others have shown an association between the use of certain materials (like rubber and plastic products) and glioma development (38). Exposure to medium-to-high-dose ionizing radiation can also be an

important environmental factor that has been proven to be unambiguously associated with glioma development in studies involving atomic bomb survivors (39) as well as children irradiated for other medical conditions (40–42). However, it must be said that results are often inconsistent among different studies.

Genetic predisposition can play a role as a risk factor for this disease. Several monogenic Mendelian diseases are associated with an increased risk of developing glioma, including neurofibromatosis 1 and 2, tuberous sclerosis, Li-Fraumeni syndrome, retinoblastoma, Turcot syndrome, Lynch syndrome, melanoma-neural system tumor syndrome and Ollier disease/Maffucci syndrome (16,43). Conversely, patients with allergic diseases (hay fever, asthma, food allergies and others) are considered to have a lower risk at developing a glioma tumor (16,43). These findings suggest that the immune system plays an important role in the development of these tumors (44).

1.2. The immune system and GBM: a complex relationship

In the past, the brain has been considered an immune-privileged organ that was physically isolated from the systemic immune system by the BBB. It was also thought that the brain lacked a connection with the lymphatic system. However, contrarily to old believes, it is currently well established that there is close and peculiar relationship between the brain and the immune system (45,46). Furthermore, diseases like GBM compromise the integrity of the BBB and the immune cells are therefore able to more freely cross the BBB and reach the brain parenchyma (47,48).

1.2.1. Heterogeneity of the immune microenvironment

Glial tumor cells can secrete multiple cytokines, chemokines and growth factors that promote the attraction of different types of immune cells such as macrophages (MFs) (49), dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) and T cells ($CD4^+$, $CD8^+$ and regulatory T cells (T_{reg})) (50,51). However, not only immune cells but also astrocytes, endothelial

cells, circulating hematopoietic progenitor cells and others can be recruited to the site of the tumor, contributing to create a unique niche, which is called tumor microenvironment (52,53).

Tumor-infiltrating immune cells play a major role in this context, since they can regulate tumor growth and development. Two sides of the same coin, immune cells can both promote or suppress tumor development, depending on the specific type of cell, on the activation status and on the interactions with other subpopulations (53).

1.2.2. Dendritic cells

Dendritic cells play an important part in the immune regulation of malignant brain tumors, since they are the main antigen presenting cells for tumor antigens (APCs). The immune reaction starts with DCs uptaking tumor antigens and migrating to the lymph nodes. After processing, the antigens are presented to the T cells in order to activate the anti-tumor adaptive immune response. The capability of tumor infiltrating DCs to migrate to the lymph nodes and to present the tumor antigens to naïve antigen-specific T cells via major histocompatibility complex (MHC) class I and II molecules represents a fundamental step in the whole picture of brain tumor immunology (54).

1.2.3. T lymphocytes

Three main types of T infiltrating lymphocytes (TILs) are present in the brain tumor microenvironment, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and $CD4^+$ $FoxP3^+$ T regulatory cells (T_{regs}) (52). The recognition of tumor antigens (presented by DCs) by $CD4^+$ T helper cells and $CD8^+$ T cells is necessary for the activation of naïve $CD8^+$ T cells into effector $CD8^+$ cytotoxic T cells. Activated effector $CD8^+$ cytotoxic T cells are then able to kill cancer cells expressing the tumor antigens they were primed for (55). Conversely, T_{regs} act as immunosuppressive agents since they can hinder the function and proliferation of $CD8^+$ T cells (56–58). $CD4^+$ TILs in brain tumors play a multifaceted role. From one side, $CD4^+$ T-helpers are critical in different aspects of the activation of the adaptive immune response, since their primary role is to recruit, activate and positively regulate effector $CD8^+$ T cells. However, under the effect of factors secreted by glioma cells, $CD4^+$ T cells can also give rise to T_{regs} and exert a strong immune-suppressive capacity, thus promoting

tumor progression (59). T_{regs} act via several mechanisms, such as the inhibition of the production of interleukin 2 (IL-2) by the $CD4^+$ and $CD8^+$ T cells and the direct suppression of their proliferation (56,57). It has been shown that blocking the T_{reg} cells in a glioma mouse model increases the amount of $CD4^+$ and $CD8^+$ T cells and potentiates their capacity to exert their cytotoxic effects. Furthermore, targeting T_{reg} cells increased the infiltration of myeloid cells, both M1 macrophages and granulocytes, into the tumor microenvironment and significantly prolonged the life of the mice (60). It is clear that during the development of malignant brain tumors, an immunological negative synergism is in place: glioma cells attract and stimulate the development of T_{regs}, which in turn stimulates immune-tolerance towards the tumor and favors tumor growth. This vicious cycle is particularly difficult to break and it represents one of the major obstacles against the generation of a positive immune response against HGGs (61).

T_{reg} related mechanisms are not the only ones acting against a proper anti-tumor immune response in GBM. Glioma cells are able to secrete anti-inflammatory and pro-tumorigenic cytokines, such as transforming growth factor β (TGF-β) and IL-10 (52,62). In addition, malignant cells lack the expression of CD80/CD86 and overexpress programmed cell death ligand 1 (PD-L1) (63). CD80/CD86 are co-stimulatory molecules which are fundamental for the final activation of the cytotoxic effect of $CD8^+$ T cells. PD-L1 stimulates its receptor (PD-1), which is overexpressed in $CD8^+$ TILs, and induces the activation of several molecular pathways which are responsible for a specific type of T cell inactivation which is called T cell exhaustion (64,65). It has been shown that PD-L1 is upregulated in GBM patients, it is expressed both on the tumor cells as on the antigen presenting microglia (66). In the majority of GBM patients investigated by Berghoff et al. (66), in 88% of newly diagnosed patients and in 72% of recurrent cases, a positive immunohistochemistry staining for PD-L1 was obtained. These results suggest that the immunosuppressive axis PD-1/PD-L1 present in glial tumors may be one of the important factors in the immune evasion mechanisms of GBM. Nevertheless, a consensus is still lacking concerning what can be defined a PD-L1 positive glioma, since different results can be obtained with different staining kits (67).

Another essential pathway contributing to the immunosuppression in GBM involves cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 is a co-inhibitory receptor present on activated $CD4^+$ and $CD8^+$ T cells that competes with the co-stimulatory receptor CD28 for binding to CD80

and CD86 (65) with a higher affinity for these ligands than CD28 itself (68). It causes suppression of antigen-specific T cell activation by interfering with the costimulatory signaling and thus inhibiting the naïve and memory T cell activation (69). Another function of CTLA-4 is the enhancement of immunosuppression by T_{regs} (70).

Expression of indoleamine 2,3 dioxygenase (IDO) can be importantly involved in the evasion of the anti-tumor immune response by GBM cells. IDO induces proliferation arrest in T lymphocytes by inducing a tryptophan (71,72). Additionally, IDO expression by GBM cells was reported to recruit more T_{regs} in the brain tumor microenvironment and correlates also in this way with an increased immune suppression (73). Expression of IDO in human glioma samples was negatively correlated with survival and higher IDO expression was seen in GBM patients compared to gliomas of lower WHO grade (73).

A last important inhibitory pathway in GBM involves the Fas/FasL apoptosis axis. When FasL binds to its receptor, Fas, it activates an intracellular signaling cascade involving caspase activation and ultimately apoptosis. Physiologically, its role is to limit the inflammatory response by inducing apoptosis of infiltrating and pro-inflammatory lymphocytes (74,75). A higher FasL expression was observed in GBM samples compared to normal glial tissue, as well as a higher caspase 8 and caspase 3 expression (76). FasL expression on glioma cells was reported to be able to induce apoptosis in activated T lymphocytes whereas FasL knockdown was able to inhibit the glioma induced T cell apoptosis *in vitro*. *In vivo*, using FasL knockdown glioma cells in immunocompetent animals resulted in a decrease in tumor volume and increase in $CD3^+$ cells (74), indicating the importance of this interaction in the evasion of immune surveillance by GBM.

Notably, in some extent the relationship between the above mentioned three different TILs has a certain prognostic value. In human patients, the number of $CD8^+$ TILs appears to be significantly lower in HGG compared to LGG while the opposite is valid for $CD4^+$ TILs (59). Importantly, T_{reg} cells have only been found in GBM patient samples and not in lower grade astrocytomas (51,59,77), indicating their specific role in the malignant phenotype of GBM. Taken together, these evidences show that the presence of a higher amount of suppressive cells a lower amount of effector cells can be related to the malignant phenotype of GBM when compared with lower grade tumors (51). However, despite some of these differences in GBM TILs that have been

shown to be related to patients' survival, they cannot be considered as true biomarkers. As an example, high $CD4^+$ TILs in combination with low $CD8^+$ TILs in GBM patients was associated with a shorter progression free survival (59). However, measuring T_{regs} in GBM patient samples, both blood and tumor tissue, showed no significant correlation with patients' survival (78).

1.2.4. Natural killer cells

Natural killer cells (NKs) belong to the innate immune system and can execute spontaneous cytolytic activity against cells in stress conditions such as viral infections and cancer. The cytolytic effect of NK cells is mediated by their degranulation and release of perforin and granzymes or by the induction of apoptosis of their target cells by activating death receptors (79). When activated, NKs also play an important role in modulating the innate and adaptive immune system by secreting several cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF) and some chemokines (79). In normal conditions, the activation of NK cells depends on the signals coming from activating and inhibiting receptors. On the surface of normal healthy cells, the presence of MHC class I molecules inhibits NK cells as a mechanism of self-tolerance. Tumor cells lose their MHC class I expression leading to a decreased activation of the NK inhibitory receptor. Furthermore, tumor cells also express ligands for the activating receptors, which activates the NK cells even more (79).

Despite the theoretical bases for NKs activity against gliomas, it remains an open question whether these cells are truly able to infiltrate the HGG microenvironment and whether they play a role in the anti-tumor immune response. Contrasting results have been published on this matter. Stevens et al. (80) reported the absence of NK cells in most glioma samples, while Yang et al. (81) reported the opposite, namely a frequent infiltration of NK cells in GBM samples. Others have reported that there are some infiltrating NK cells present, but they only account for 2,11% of total tumor infiltrating immune cells (82). Independently of their abundancy, it was shown that *ex vivo* cultured human GBM cells with stem cell-like properties were recognized and killed by NK cells. Interestingly, these cells were resistant to freshly-isolated NK cells but they were susceptible to NK cell mediated cytotoxicity by both autologous and allogeneic NK cells after IL-2 or IL-15 mediated activation (83).

1.2.5. B lymphocytes

The exact role of B lymphocytes in the development of cancer is not completely understood yet. The tumor microenvironment contains a very heterogeneous population of B lymphocytes and it is possible that functionally distinct subsets of B cells contribute to both pro- and anti-immune responses. The balance between these responses probably determines whether the B cells exercise a pro- or an anti-tumor effect (84). Some studies have reported the presence of B cells in gliomas but the fraction of these cells in the total amount of infiltrating immune cells was very low (53,81). Candolfi et al. (85) reported that B cells can act as APCs for T cells in a GBM model, indicating the possible importance of so-called tumor infiltrating B cells (TIL-Bs) in the T cell mediated anti-tumor activity. By their APC action, TIL-B cells may be inducing $CD4^+$ T cell-dependent $CD8^+$ memory T cells and thus controlling tumor invasion, spread and metastasis (86). On the other hand, TIL-Bs have been shown to suppress the development of an anti-tumor immune response since a specific subpopulation of regulatory B cells (B_{regs}) has been described. This subset of B cells produces immunosuppressive cytokines, such as TGF-β and IL-10, therefore suppressing the anti-tumor immune response (84). By these means, B_{regs} can suppress the activation of T cells or facilitate their conversion in T_{regs} (87). Glioma cells derived from human biopsies have been shown to express surface proteins that induced activated B cells to differentiate into this B_{regs} consequently able to suppress $CD8^+$ T cell proliferation and to induce T_{regs} differentiation (88). Besides these early evidences, the actual role of B cells in the cancer microenvironment still requires further investigation in order to determine which is their specific function is and whether they can be targeted with specific therapies.

1.2.6. Macrophages

Macrophages are part of the innate immune system and are generally classified in two different subsets: the *classical* M1 macrophages and the *alternative* M2 macrophages (89). Upon activation, M1 macrophages produce several pro-inflammatory cytokines such as TNF-α, IL-12, IL-6 and IL-23. They also express high amounts of MHC class I and II molecules, necessary for antigen presentation. Given these features, M1 macrophages are usually considered to provide a pro-inflammatory and anti-tumor response. The conversion of M1 macrophages towards an M2

phenotype can be stimulated by cytokines such as IL-4, IL-13, IL-10 and by TLR activation (89). M2 macrophages produce a variety of anti-inflammatory cytokines such as TGF-β, IL-10 and arginase-1 (ARG-1) (90). For this reason, M2 macrophages are considered to have a more anti-inflammatory and pro-tumorigenic activity.

In GBM, tumor associated MFs (TAMs) represent up to 50% of the cells of the tumor mass, including both infiltrating MFs and resident brain microglia, constituting approximately 85% and 15% of the total TAM population respectively (49). Brain microglia are glial cells that function as APCs inside the brain. Microglia are morphologically indistinguishable from bone marrow derived MFs. Traditionally they can be distinguished from one another based on CD45 expression using fluorescence-activated cell sorting (FACS) analysis, as microglia are $CD45^{Lo}$ and infiltrating MFs are $CD45^{Hi}$ (49). The infiltrating MFs are recruited to the tumor in early stages of its development and are preferentially localized near the vascular areas, while the resident microglia are more localized in other areas of the brain in the proximity of the tumor (49). TAMs are thought to be more closely related to the M2 phenotype and thus demonstrate a more pro-tumorigenic activity (91). Therefore, they are usually associated with a poor prognosis (90). TAMs have already been shown to promote tumor progression directly, by stimulating angiogenesis in HGG models and lymphangiogenesis in other types of cancer such as lung adenocarcinoma, and indirectly, by contributing to block the immune reaction against the tumor (90,92,93). They can induce immune suppression via secretion of specific anti-inflammatory cytokines such as TGF-β, which has an important immunosuppressive role in both the innate and adaptive immune system. In the innate immune system, it inhibits the cytolytic activity of NK cells expressing natural killer group 2D (NKG2D), one of the activating receptors, against the tumor cells (94). Furthermore, it is auto-stimulating as it enhances the conversion from M1 to M2 macrophages, which in turn causes more TGF-β secretion (95). In the adaptive immune system, TGF-β inhibits the $CD8^+$ T cell activity, since TGF-β specifically inhibits the production of perforin, granzyme A and B, FasL and IFN-γ which are usually responsible for $CD8^+$ T cell mediated tumor cytotoxicity (90,96). Also DCs migration is inhibited and apoptosis is promoted by TGF-β, therefore the whole activation of the adaptive immune response is dampened (90,95). However, contrasting data are available concerning the role of M1 and M2 macrophages in HGG. In a glioma mouse model, depletion of MFs resulted in

increased glioma growth, indicating that (at least in some cases) TAMs can slow down glioma tumor progression and exert a beneficial role in gliomas instead of promoting immune-evasion (97). So far, the mechanisms explaining this results still remain unclear (53,97).

1.2.7. Myeloid-derived suppressor cells

Another immune suppressive mechanism exploits the functions of aberrant and immature myeloid cells, named MDSCs. Myeloid-derived suppressor cells represent a very heterogeneous population of immature myeloid cells derived from bone marrow and include myeloid progenitors and MF, DC and granulocyte precursors. Myeloid-derived suppressor cells have the ability to suppress NK and T cell function, inhibiting the anti-tumor immune response (98). They consist of a very heterogeneous cell population based on morphological differences and surface staining and can be divided into two subsets. In mice, the monocytic MDSCs (mMDSCs) are characterized by the phenotypical expression of lymphocyte antigen 6 complex locus C (Ly6C) while the granulocytic MDSCs (gMDSCs) are characterized by Ly6G expression. In humans mMDSCs and gMDSCs are phenotypically characterized as $CD14^+$ and $CD15^+$ respectively (99–101).

Myeloid-derived suppressor cells are normally not found in the circulation of healthy individuals, but they tend to accumulate in the blood and tumor tissue of cancer patients (102,103). They have been proven to suppress the anti-tumor T-cell response in different ways as they have an inhibitory effect on both T cell function and proliferation; however, the mechanism behind this suppressive effect is not completely unraveled yet (102). In GBM patients, the level of circulating MDSCs isolated from peripheral blood mononuclear cells (PBMCs) was shown to be higher than healthy donors and patients with other types of cancers such as melanoma, bladder carcinoma and renal cell carcinoma (99), indicating a possible involvement in GBM immune biology. The contribution of these MDSCs to T cell suppression has been examined by evaluating the IFN-γ production by $CD3^+$ T cells. Glioblastoma patient derived T cells displayed a reduced IFN-γ production after stimulation with anti-CD3/CD28 antibodies compared to healthy donors, and TNF-γ production was successfully restored in these T cells after MDSC depletion (99). Glioma animal studies have reported the role of CCL2 in the recruitment of both T_{regs} and mMDSCs (104). $CD11b^+$ MFs and microglia were found to be the major source of CCL2, indicating a link between

different immune populations and their cooperation in maintaining the immune suppressive microenvironment of GBM (104). The importance of CCL2 pathway was also confirmed by human studies, which demonstrated that a higher CCL2 expression in GBM samples is associated with a significantly decreased median survival compared to a low CCL2 expression (104).

Besides CCL2, other complex mechanisms are involved in the immune suppressive properties of MDSCs (101). One of these is the production of nitric oxide (NO) and reactive oxygen species (ROS), causing respectively T cell apoptosis and impairment of T cell receptor (TCR) expression on CD8+ T cells (therefore rendering them unresponsive for antigen-specific activation) (105). By these means, tumor cells become resistant to antigen-specific TILs (101). A second mechanisms is the involvement of ARG-1, also shown to be expressed by MDSCs derived from GBM patients (50). Arginase-1 is responsible for metabolizing L-arginine into L-ornithine. This arginine depletion ultimately results in an arrest of the T cells in G_0-G_1 phase of the cell cycle (101). In glioma models, MDSCs have also been proven to suppress NK cell cytotoxicity by inhibiting both IFN-γ and the activating receptor NKG2D (101,106). The last essential MDSCs' suppressive mechanism is their capability to recruit T_{regs} to the tumor microenvironment (101). Taken together, all these results support the hypothesis that activation of T cells and NK cells is suppressed by MDSCs in GBM patients, making them one of the most important players in the GBM suppressive microenvironment.

1.3. Conventional therapies: an insufficient treatment

Glioblastoma is difficult to treat due to the presence of a very heterogeneous cell population, able to develop different resistance mechanisms. For this reason, GBM therapy combines multiple approaches (5). As already mentioned, the standard therapy schedule for GBM patients consists of macroscopically complete surgical resection of the tumor (when possible) followed by RT combined with adjuvant and concomitant TMZ as it is defined by the Stupp protocol (12,107).

1.3.1. Surgical resection

Surgical resection is an important first step in the treatment of GBM patients to provide the right diagnosis and reduce the mass effect due to the tumor mass. In terms of outcome, the extent of resection is associated with a better prognosis for both newly diagnosed and recurrent GBM patients (108,109). However, the main challenge remains the preservation of neurological functions. As a consequence, a complete resection is not always possible. Several methods can help in maximizing the safety and precision of the procedure, including functional MRI, intraoperative imaging and 5-aminolevulinic acid (5-ALA) fluorescence-guided surgery (14,110).

1.3.2. Radiotherapy

In 1978, a randomized controlled clinical trial proved for the first time a survival benefit of RT in anaplastic glioma patients (111). In this trial, a cumulative dose of 50 to 60 Gy delivered in 2 Gy fractions for a period of 5 to 7 weeks was able to prolong median survival of patients with 22 weeks (median survival: 36 weeks in RT group and 14 weeks in supportive care group). In 1979, the same protocol was shown to prolong survival also of GBM patients (increase in median survival of 24 weeks when receiving 60 Gy) and a dose-response relationship was described between the cumulative dose of radiation and survival (112). In the following decades, several clinical trials have been performed but the radiation dose remained approximately the same since a higher dose was shown to increase side effects with no additional survival benefit (113,114).

An important improvement in the field of RT consisted in the progressive limitation of the irradiated volume, aiming at reducing the damage of normal brain tissue and maximizing the radiation delivered to the tumor cells. Since several studies showed that tumor recurrence mostly often occurs within a margin of two to three cm around the surgical cavity, currently a more focused treatment is performed instead of whole brain irradiation (115,116).

1.3.3. Chemotherapy

The most used chemotherapeutic agent in HGGs is TMZ, an alkylating agent that causes DNA damage by adding alkyl groups to the purine bases of the DNA, namely O^6-guanine, N^3-

adenine and N^7-guanine (117,118). This triggers the DNA mismatch repair mechanisms and ultimately causes cell apoptosis. After successful preclinical studies and Phase I clinical trials, in 2005 a randomized Phase III study promoted by the European Organization for Research and Treatment of Cancer (EORTC) and conducted by Stupp and colleagues finally demonstrated the superiority of RT associated with TMZ compared to RT alone in GBM patients (107,119–121). After this study, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) approved the use of TMZ use for GBM.

As already mentioned, TMZ action can be partially counteracted by MGMT, which is able to remove the alkyl groups from the DNA to provide a resistance against TMZ. High levels of MGMT expression are associated with TMZ resistance and poorer prognosis (122). On the other hand, epigenetic silencing of the MGMT gene by its promotor's methylation can serve as a mechanism to decrease MGMT activity in tumor cells (117). The evaluation of MGMT promotor methylation is currently being used as a predictive marker for the GBM response to TMZ.

The only other FDA-approved chemotherapeutic agent for GBM is Carmustine (BCNU), a DNA-alkylating agent that can be administered through polymer wafers that are positioned in the tumor bed after surgical resection to exert more local chemotherapeutic effects. However, BCNU wafers have proven conflicting results: in some trials survival of patients was (slightly) improved with no increase of adverse events, in other studies the survival benefit was not significant and the rate of adverse events was nog negligible. For these reasons, the use of BCNU wafers is still a matter of debate (123–125).

1.3.4. Immune effects of standard therapy

Standard GBM therapies have all direct or indirect effects on the immune system and can thus modulate the anti-tumor immune response, either by stimulating or inhibiting it.

It has been demonstrated that irradiation of tumor tissue induces the release of danger-associated molecular patterns (DAMPs) from the tumor cells. Dendritic cells are able to recognize these patterns and present them to naïve T cells, thereby stimulating the adaptive immune response against the remaining tumor cells (126), a process called immunogenic cell death (ICD) (127). For RT, a distinction has to be made between whole-body and focal irradiation, as they have

different effects on the anti-tumor immune response. Immune cells, especially T and B lymphocytes and NK cells, are among the most radiosensitive cells in our body (128,129). Therefore whole-body irradiation has a rather immune suppressive effect because it also affects important organs involved in the production, differentiation and maturation of immune cells, such as the spleen, the thymus and the bone marrow (130). A more focal irradiation strategy, as it is applied with GBM patients, doesn't induce this systemic immune suppressive effect and has a more immune stimulatory effect by the mechanisms of ICD (131).

Regarding the immune effects of TMZ, it was described that one of the major side effects of this treatment is myelo-toxicity, ultimately causing systemic lymphopenia (132). This can be partially explained by the low levels of MGMT in hematopoietic progenitor cells in the bone marrow, which renders bone marrow derived cells more susceptible to DNA damage caused by TMZ (133). However, contrarily to this suppressive effects of TMZ, it has been recently demonstrated that an increased antigen-specific T cell response can be present during the recovery phase which usually takes place after the stop of TMZ treatment (134,135).

Potent immune responses have been reported in GBM patients receiving a combination therapy of an EGFRvIII-targeted peptide vaccine and high myelo-ablative doses of TMZ. The strong initial myelosuppression caused by TMZ was able to induce a bigger immune stimulation to potentiate the activity of the vaccine (134), indicating that TMZ can have a beneficial immune stimulatory effect in combination with emerging immune therapies as similar results have been published in a preclinical murine GBM model with a DC vaccine (134,136).

Furthermore, multiple studies have been done investigating the effects of RT and TMZ combination treatment on the circulating immune cells in GBM patients. A six week treatment schedule significantly increased the proportion of $CD4^+$ T lymphocytes with a T_{reg} phenotype in peripheral blood four weeks after completion of the therapy, although it had no effect on the absolute number of T_{regs}. These results suggest that circulating T_{reg} cells are less sensitive than other cells of the $CD4^+$ T cell population to the cytotoxic effects of RT and TMZ in GBM patients. Furthermore, results of the same study indicated that the numbers of NK and $CD8^+$ effector cell counts were decreased in peripheral blood after the RT and TMZ treatment schedule (137). From this study, it can be concluded that the combination of RT with TMZ induces a shift towards

immune suppression in the immune cell compartment of the peripheral blood and this should be taken into account when setting up experiments regarding immune therapy. However, sufficient data about the immunological effects on the tumor microenvironment are currently still lacking.

It is clear that both radiation and chemotherapy effects are not limited to the direct killing of GBM cells, but complex immune modulating effects are also in place. However, the available data about the effects on the tumor immune microenvironment are still very limited and a more extensive preclinical and clinical evaluation is necessary to fully unravel these mechanisms. This immunological evaluation is crucial in order to be able to effectively include new emerging treatment strategies, such as immunotherapy, into the treatment schedule of GBM patients.

1.4. The potential of immunotherapy in GBM

At present, GBM is still considered an incurable disease as GBM patients face a median survival of 15 months following an aggressive combination of therapies including maximal safe surgical resection and adjuvant radiation and chemotherapy (12). Therefore, development of new treatment strategies is needed to improve patients' prognosis. In this regard, immunotherapy is one of the most interesting novelties of the last decades. The main goal of immunotherapy is to induce a shift in the immune biology of the tumor from suppression towards activation, in order to exploit the immune system of these patients as an effective weapon against the tumor.

1.4.1. Different immunotherapeutic strategies

Different immunotherapeutic strategies exist, either with an active or passive approach. In the category of passive immunotherapy, either monoclonal antibodies (mAbs) can be used, coupled to a toxic compound, or adoptive cellular transfer of immune cells, which includes lymphocyte-activated killer (LAK) cells and cytotoxic T lymphocytes (CTLs), can be performed.

Alternatively, active immunotherapy stimulates the patient's native immune system in providing the appropriate immune response against the tumor. One active immunotherapy approach consists of the development of DC vaccines. Autologous immature DCs can be isolated

and primed against a wide range of tumor antigens *ex vivo* in order to effectively activate the adaptive T cell response against the tumor cells when injected back into the patient (138,139). A second active approach can be the use of immune checkpoint inhibitors. The balance between co-stimulatory and co-inhibitory molecules is essential in mediating immune homeostasis and maintaining an appropriate immune response. In cancer, these immune checkpoints are often modulated to create an immune suppressive microenvironment and even promote tumor progression. To reverse this process, immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1/PD-L1 can be used in the battle against cancer.

1.4.2. Checkpoint inhibitors in GBM

In a preclinical study using a glioma murine model, also the efficacy of the immune checkpoint strategy has been shown (140). Long term survival of the mice was obtained for the majority of the treated animals, normal $CD4^+$ T cell count was established and no further increase in T_{reg} population was observed. These PD-L1/PD-1 inhibitors have been studied in murine GBM models in combination with stereotactic RT. This combination was able to double the survival and to induce long term survival in a fraction of the mice (141). Furthermore, good preclinical results have been obtained with the combination of anti-CTLA-4 and anti-PD-1 mAbs (142). Based on very promising preclinical results, clinical trials have been set up in an attempt to translate these results to actual patients. Unfortunately, the results of these trials haven't been satisfactory. Only recently, the first large scale randomized phase III clinical trial investigating the effects of Nivolumab, an anti-PD-1 monoclonal antibody, has failed to show an increase in overall survival for recurrent glioblastoma patients (143,144). Several clinical trials are currently still ongoing with both anti-PD-1 antibodies (Nivolumab and Pembrolizumab) alone or in combination with anti-CTLA-4 antibodies (Ipilimumab) or anti-VEGF therapy (Bevacuzimab), of which no results have been communicated yet. An overview of currently ongoing clinical trials concerning anti-PD-1 treatment is given in Table 1. Despite some of the results currently available show a potential clinical impact, important improvements (most likely requiring further preclinical investigations) need to be performed before that this treatment strategy can show relevant effects in clinic.

Table 1: Overview of clinical trials involving anti-PD-1 checkpoint inhibitors. GBM = glioblastoma, OS = overall survival, MTD = maximal tolerable dose, DLT = dose limiting toxicity, PFS6 = 6 month progression free survival, RP2D = recommended phase 2 dose, NPR = non-progression rate, MGMT = methylguanine methyltransferase

Clinical trial	Study type	Study population	Treatments	Primary endpoints	Current status
NCT02530502	Phase I/II	Newly diagnosed GBM	Pembrolizumab in combination with standard treatment	DLT and PFS6	Active, not recruiting
NCT02337686	Phase II	Recurrent GBM	Pembrolizumab before surgical resection and continuing after surgery	PFS6 and immune effector/T_{reg} ratio	Active, not recruiting
NCT02337491	Phase II	Recurrent GBM	Combining Pembrolizumab with Bevacuzimab compared to Pembrolizumab alone	MTD, DLT and PFS6	Active, not recruiting
NCT02550249	Phase II	Newly diagnosed and recurrent GBM	Neoadjuvant Nivolumab	Expression levels of PD-L1	Completed, awaiting results
NCT02667587 (Checkmate 548)	Doubled blinded randomized phase III	Newly diagnosed MGMT methylated GBM	Standard treatment combined with placebo or Nivolumab	OS	Recruiting
NCT02617589 (Checkmate 498)	Unblinded randomized phase III	Newly diagnosed MGMT unmethylated GBM	Standard treatment vs radiotherapy combined with Nivolumab	OS	Recruiting
NCT02017717 (Checkmate 143)	Phase III	Recurrent GBM	Effectiveness of Nivolumab compared to Bevacuzimab and of Nivolumab with or without Ipilimumab	OS and safety and tolerability	Nivolumab failed to meet primary endpoints

2. Rationale and objectives of the research

In the last years, no experimental treatments have been able to significantly prolong the survival of GBM patients, even the most recent immunotherapeutic strategies. In this regards, we believe that one of major issues has been related to the clinical combination schedules that are barely supported by robust preclinical data.

The experimental treatment has clinically been administered after standard radio-chemotherapy or at tumor recurrence, but without previously assessing the efficacy of this combination schemes in a murine setting. We believe that to synergistically combine conventional and new experimental treatments and to fully exploit their therapeutic potential it is necessary to base such combinations on rational preclinically based data.

Since chemotherapy, radiotherapy, and immunotherapy are all able to modulate the immune biology of brain tumors, we hypothesize that this common feature could be used to drive their rational and synergistic combination; however, very little information is currently available in this field. In this project, we analyzed the modification of the tumor-infiltrating immune cells following the administration of the above-mentioned therapies, alone and in combination. The aim will be to create new combination strategies aimed at reducing the immune suppression and maximally potentiate the immune activation against the tumor. By using this immune-driven approach in the design of combination schedules, we hope to ultimately close the translational gap currently present and to pave the way for a successful treatment of GBM patient

3. Material and methods

3.1. Cell culture

CT-2A mouse astrocytoma cells were obtained from professor Tom Seyfried (Department of Biology, Boston College Chestnut Hill, MA, USA). CT-2A cells were cultured as monolayers (ML) in Dulbecco's Modified Eagle Medium (DMEM; Thermofisher), enriched with 10% heat-inactivated fetal calf serum (FCS; Thermofisher). Subsequently, cells were incubated in a T75 flask with filter cap at 37°C in humidified air and 5% CO^2. To generate CT-2A NS cells, ML cells were enzymatically dissociated with Accutase (Sigma Aldrich). Cells were washed and plated in a T75 filter flask at a concentration of 1 x 10^5 living cells/ml in a total of 20 ml of DMEM medium with F-12 nutrient mixture (DMEM/F-12; Thermofisher) enriched with epidermal growth factor (EGF; Thermofisher) and fibroblast growth factor (FGF; Thermofisher) both at a final concentration of 20 ng/ml, and B27 supplement (Thermofisher) at 2%. Cells were incubated at 37°C in humidified air and 5% CO^2. On day 3 after plating, an additional 2 ml of DMEM/F-12 medium supplemented with 4 µl of EGF and 4 µl of FGF was added to the flasks. On day 6 after plating, floating NS cells were collected, enzymatically dissociated with Accutase, washed and re-plated at a concentration of 1 x 10^5 living cells/ml in a total of 20 ml of DMEM/F-12 medium. Another supplement of growth factors was added on day 8 as described previously. On day 11, floating NS were collected and prepared for *in vivo* experiments.

3.2. In vivo model

3.2.1. Animals

The animals used for this experiment were female adult C57BL/6J mice purchased from Harlan (Horst, The Netherlands). Mice were housed in individually ventilated cages kept at a constant temperature (21 ± 2°C), humidity (50 ± 10%) and light cycle (12h/12h light/dark). Permanent access to food and water was provided. All animal experiments were all approved by the ethics committee of the Katholieke Universiteit Leuven (project 089/2015 and amendments) in correspondence with international guidelines.

3.2.2. Orthotopic HGG mouse model

To generate the orthotopic HGG model, day 11 floating CT-2A NS were collected and enzymatically dissociate in order to obtain a single cell suspension. Each mouse received intacranially 5 x 10^3 living cells in 4 µl of DMEM/F-12 culture medium. An amount of 6 µl/g body weight of anesthesia composed by a mixture of 18,75 mg/ml ketamine (Pfizer) and 0,125% xylazine hydrochloride (Bayer), was injected intraperitoneally (IP) to the mice. On the head of the mice, 2% lidocaine hydrochloride cream (AstraZeneca) was applied and mice were placed in prone position on a stereotactic frame (Kopf Instruments). After disinfection of the skin, a longitudinal 1,5 cm incision was made. A hole was drilled through the skull 0,5 mm anterior of the bregma and 2,5 mm lateral from the midline. Cells were slowly injected 2,5 mm below the dura mater over a period of 4 minutes, with a 26 gauge syringe (Hamilton). In order to prevent cell inoculation in the direction of the ventricle, the bevel of the needle was directed laterally during the injection. The needle was left in its position for another 4 minutes before slowly retracting it. The hole in the skull was sealed with sterile bone wax before suturing the incision with silk stitches. When awake, mice were put back in their cages where they had free access to food and water.

3.3. Treatments

In a first set of experiments, mice were randomized to receive RT, TMZ or the immune checkpoint inhibitor anti-PD-1 as single treatments. Untreated mice were used as controls. As showed in Figure 1, all treatments started on day 12 after tumor inoculation and dosages were chosen based on pilot experiments previously performed (Riva, Wouters, Coosemans et al, unpublished data). For RT, external beam image-guided arc irradiation was performed using a Small Animal Radiation Research Platform (SARRP; Xstrahl Life Sciences) with a dosage of 4 Gy delivered in one single dose. On the day before irradiation, MRI was performed following the protocol described below. The MRI scan was merged with the CT scan performed on the day of irradiation to determine the radiation isocenter inside the tumor area for each mouse. For TMZ, Temodal 20 mg capsules (Merck Sharp & Dohme Limited) were dissolved in phosphate-buffered

saline (PBS; Thermofisher) at a concentration of 10 mg/ml. To increase solubility, 10 mg/ml of L-Histidine (Sigma Aldrich) was added and a sonication bath was used for 30 minutes. Administration of TMZ was done via oral gavage. The dosage schedule consisted of 4 doses of 50 mg/kg on alternate days. The anti-PD-1 antibody used for these experiments was kindly provided by Bioceros BV (The Nederlands) and manufactured as follows. The anti-PD-1 hybridoma (Clone RPMI-14) was cultured in Iso-cove's Modified Dulbecco's Medium (IMDM; Lonza) containing 1% fetal bovine serum (FBS) and was sterilized by a 0,2 mm filtration. Mice received 3 single doses of 100 µg anti-PD-1 administered on alternating days through IP injection.

In a second set of experiments, mice were randomized to receive single and combination treatments including two or three different therapies (Figure 2). For the combination treatments, mice were randomized into different combination treatment schedules including both the standard treatments (RT and TMZ) and the anti-PD-1 treatment (Figure 2) at the same dosages as described for the single treatment experiments.

3.4. Experimental design

3.4.1. Single therapies

Two sets of experiments were designed to assess how several anti-GBM treatments influence the populations of immune cells which are present in the brain tumor microenvironment. Three different treatments have been evaluated: RT, TMZ and anti-PD-1. In the first set of experiments, the effects of these agents as single treatments were evaluated as shown in Figure 1. A total of 40 mice was inoculated with a cell suspension of 5 x 10^3 CT-2A neurosphere (NS)-derived cells. Ten mice received RT (4 Gy single dose), 10 received TMZ (4 x 50 mg/kg) and 10 received anti-PD-1 (3 x 100 µg). Ten mice were used as controls and did not receive any type of treatment. In each of these groups, five mice were euthanized for immunological analysis on day 19 post tumor cell inoculation (at completion of all treatments) and five were monitored for survival analysis. All mice received MRI scans before and after completion of the treatment (day 11 and 19 post tumor cell inoculation) to assess tumor volume and its variations.

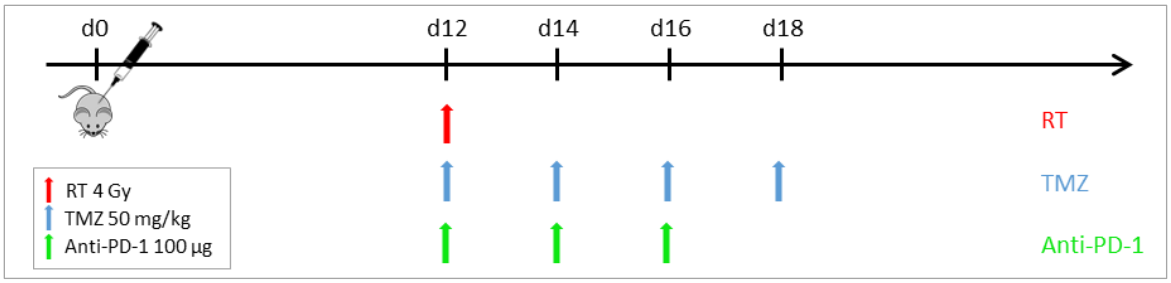


Figure 1: Treatment schedule of single treatment experiments. Mice received either radiotherapy (RT, red arrow) with a single dose of 4 Gy through external beam image-guided arc irradiation, Temozolomide (TMZ, blue arrow) with four administrations of 50 mg/kg through oral gavage or anti-PD-1 (green arrow) with three administrations of 100 µg via intraperitoneal injections.

3.4.2. Combinatorial approach

In the second set of experiments, the same treatments were evaluated in several combination schedules. In total 85 mice were used for this purpose, including ten mice receiving RT, ten mice receiving TMZ, ten mice receiving anti-PD-1 and 50 mice receiving five different treatment combinations (ten mice in each combination treatment group) as displayed in Figure 2. Five mice per group were used for survival analysis and 5 mice were used for immune monitoring on day 28 post tumor cell inoculation (after completion of all treatments). The remaining five mice were used as untreated controls for survival experiments. No untreated mice were included in the immune monitoring part of the experiment for practical reasons as control mice do not survive long enough to reach the day of immune evaluation. Also in this second set of experiments, MRI was performed before and after completion of treatments (day 11 and 28 post tumor cell inoculation). When assessing immunological effects, treatment groups were divided in two different subanalysis. The first analysis compared RT, RT – TMZ and RT – TMZ – anti-PD-1 treated mice to assess the impact of addition of both TMZ and anti-PD-1 to the treatment schedule as it is currently been done in clinical trials, while in a second analysis a comparison between all triple combination schedules (also including RT – TMZ and RT – TMZ – anti-PD-1) was made to investigate whether the moment of administration of anti-PD-1 could influence outcome.

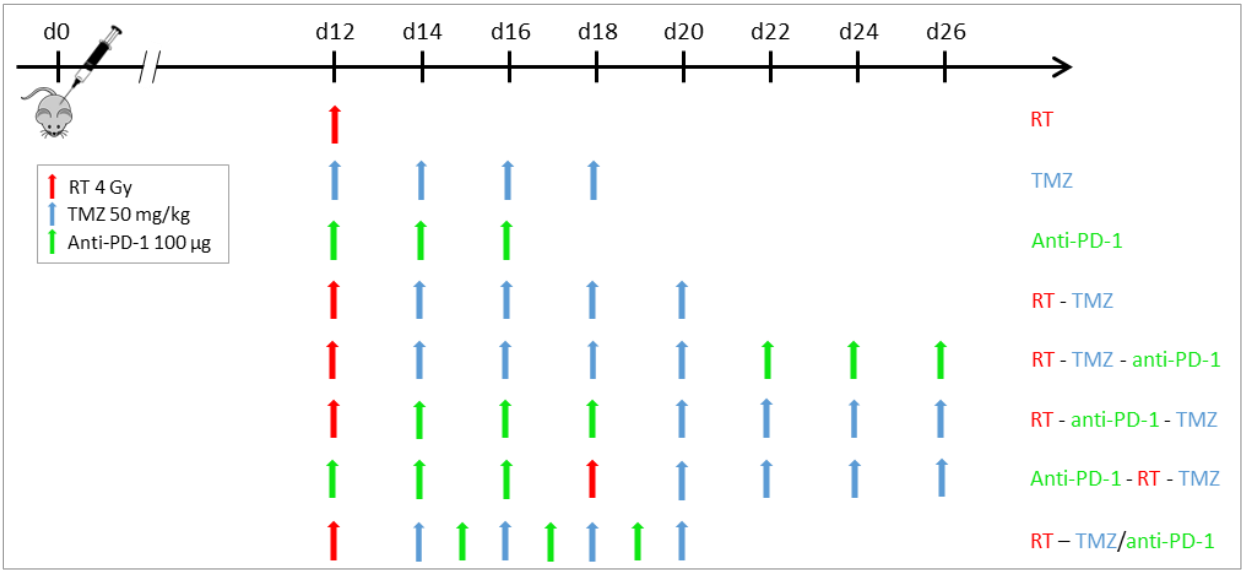


Figure 2: Treatment schedules of combination experiments. Mice received either radiotherapy (RT, red arrow) with a single dose of 4 Gy through external beam image-guided arc irradiation, Temozolomide (TMZ, blue arrow) with four administrations of 50 mg/kg through oral gavage, anti-PD-1 (green arrow) with three administrations of 100 µg via intraperitoneal injections as single treatments or combined in different schedules.

3.5. Readouts

3.5.1. Survival

After tumor cells inoculation, mice were followed measuring their weight and neurological symptoms with a minimum of 3 times a week. Mice were euthanized by cervical dislocation when they reached 80% of their initial weight or based on the severity.

3.5.2. Tumor volume

3.5.2.1. Magnetic resonance imaging

For MRI, a 7T Biospec small animal MRI system (Bruker BioSpin, Rheinstetten, Germany) was used with a 7 cm linearly polarized resonator for transmission and an actively-decoupled dedicate mouse brain surface coil for receiving (Rapid Biomedical, Rimpar, Germany). Image acquisition was performed with Paravision 6.0 software (Bruker BioSpin, Rheinstetten, Germany).

Mice were anesthetized with isoflurane (Baxter, Eigenbrakel, Belgium) in a 70:30 nitrous oxide/oxygen mixture at 3-4% for induction and 1,5-2% for maintenance. During the MRI acquisition, breath rate and body temperature were monitored using a BioTrig system (Bruker BioSpin, Rheinstetten, Germany) Breath rate was kept at 80-100/minute and body temperature was kept constant at 37°C with a custom made system using heated water.

A rapid acquisition relaxation enhancement (RARE) T2-weighted sequence with a RARE factor of 8 averages and TR/TE of 2843.532/35 ms was used. Images covered an area of 25×25 mm with a matrix size of 256×256 pixels. A total of 25 coronal-oriented slices of 0.5 mm thickness and no slice gap were acquired, covering the entire brain from the olfactory bulbs to the brainstem. To avoid inter-slice cross-talk, an interlaced scheme was applied. After the procedure, mice were put back in their cages for recovery where they had free access to food and water.

3.5.2.2. Volume calculation

The tumor area was manually delineated on each slide using the public domain software ImageJ (http://rsb.info.nih.gov/ij) as showed in Figure 3. The field-of view of the MRI slices was 25x25 mm and the matrix size was 256x256 pixels, therefore the resulting pixel density was 104,8576 pixels/mm^2. Using these values, tumor areas were converted from pixels to mm^2. Volumetric assessment was then obtained by multiplying the tumor areas of each slice by the slice thickness and summation.

3.5.2.3. Responders and non-responders

For both single and combination treatment experiments, mice were divided in responders and non-responders based on their fold increase in tumor volume before/after treatment. As a cut-off value for this stratification, the smallest tumor volume of the control group (untreated mice) on day 19 after tumor inoculation was used (6,56). For practical reasons, since no control mice survive long enough to reach day 28 after tumor inoculation, in the combination experiments the same cut-off value was used to distinguish between responding and non-responding tumors. A schematic representation of stratification between responders and non-responders is shown in Figure 3.

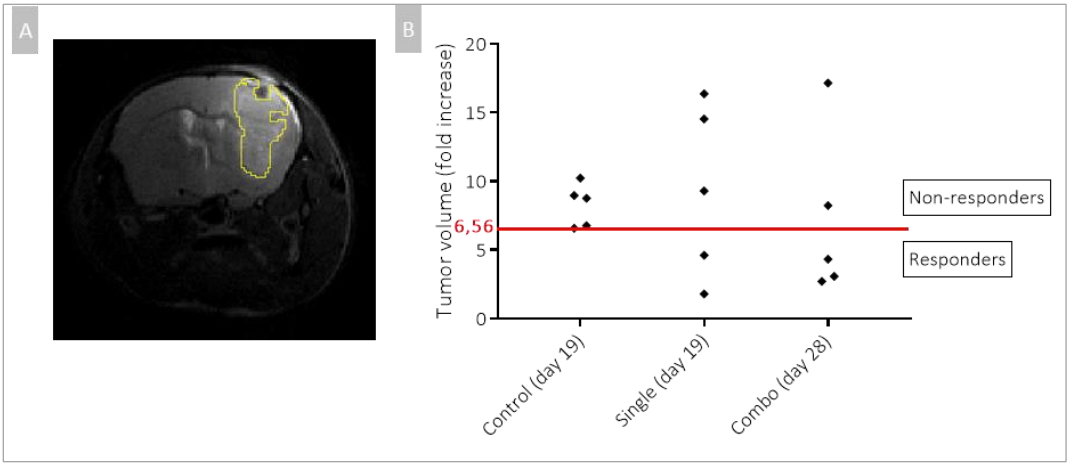


Figure 3: Volume calculation using ImageJ software and subdivision between responders and non-responders based on tumor volume. (a) Tumor volume calculated by manually delineating the tumor area on each slice as shown here, **(b)** Stratification between responders and non-responder based on fold increase in tumor volume on day 19 (control and single treatments) and 28 (combination treatments) compared to day 11 post tumor inoculation.

3.5.3. Immune monitoring

3.5.3.1. Transcardial perfusion and harvesting of the brains

Mice were deeply anesthetized with an IP injection of 50 mg/kg Pentobarbital (Lundbeck) and were subsequently placed in supine position. A 2 cm horizontal incision was made in the previously disinfected skin on the upper part of the anterior abdominal wall. The diaphragm was opened from below causing lungs collapse. The ribs were cut on both sides of the sternum and the thorax was open to expose the beating heart. A 15 gauge needle, connected to a custom made PBS perfusion system, was carefully inserted in the left ventricle. A small incision was made in the right heart auricola and mice were perfused with PBS for 5 to 10 minutes at a constant pressure of 80 mmHG until clear fluid was seen draining out of the heart. Clearance of the liver was considered an essential sign for successful perfusion.

The head was removed from the body and skull was exposed by a midline head incision from the neck to the nose. Small scissor blades ware used to cut the cranial vault at the midline from the foramen magnum to the lambda. The bone of the posterior fossa was removed with micro-forceps, exposing the cerebellum, followed by removal of the petrosum bone on both sides. A cut was made along the superior sagittal suture to remove the bone over the telencephalon.

Subsequently, a cut was made from the orbital cavity to the nose that allowed the removal of the bone covering the olfactory bulbs on both sides. The whole brain including the olfactory bulbs was carefully removed from the skull with micro-dissectors and kept in 1,5 ml cold medium consisting of Roswell Park Memorial Institute 1640 medium (RPMI; Thermofisher) enriched with 10% FCS and 0,5% ethylenediaminetetraacetic acid (EDTA; Thermofisher). Brains were kept on ice until further processing for immune cell isolation.

3.5.3.2. Generation of single cell suspension and immune cell isolation

Brains were minced into small pieces with a scalpel. Subsequently, samples were incubated for 10 minutes at 37°C with a mixture of 1 mg/ml Collagenase I (Thermofisher), 2 mg/ml Dispase I (Thermofisher) and 100 µg/ml DNase I (Sigma Aldrich) for enzymatic digestion. Brain tissue was homogenized with a Pasteur pipet and pushed through a 70 µm cell strainer in order to obtain a single cell suspension.

Tumor-infiltrating immune cells were isolated from myelinated cells and other elements by means of a Percoll gradient. Full-brain cell pellets were carefully resuspended in 10mL 25% Percoll (Sigma Aldrich) kept at 37°C and centrifuged (520g, 37°C, acceleration 5, deceleration 0, 20 minutes). This allowed to pellet the myelinated cells on top and the immune cells at the bottom of the Percoll. The myelinated cells and the Percoll were carefully removed with a transfer pipet and the remaining cell pellet of immune cells and residual red cells was washed in Hank's Balanced Salt Solution (HBSS; Thermofisher) supplemented with 10% FCS and 0,5% EDTA in order to eliminate any Percoll residues. Cells were then incubated with red blood cell lysis buffer (Thermofisher) for 10 minutes at room temperature. After washing, the cells were resuspended in PBS, transferred to a FACS tube and counted with ABX Micros 60 (Horiba).

3.5.3.3. Flow cytometry staining and acquisition

According to the stage of the tumors, a variable number of immune cells ranging from 2 to 18 x 10^6 was obtained from each tumor-bearing brain. From each sample, 1 to 3 x 10^6 immune cells were collected and resuspended in 100 µl PBS for both MF/MDSC and T cell staining.

Cells were incubated for 10 minutes at 4°C with anti-CD16/CD32 (Thermofisher) for Fc receptor blocking. After washing with PBS, cells were stained with fluorophores-conjugated antibodies. Table 2 and 3 show the target molecules and the related antibodies for MFs/MDSCs and T cells, respectively. First, samples were stained with antibodies targeted against cell surface epitopes for 30 minutes at 4°C. After washing, cells were resuspended in 100uL of PBS and incubated for 30 minutes at 4°C with 0,125 µl of Fixable Viability Dye eFluor 780 (Thermofisher). Cells were then washed and cells membranes were permeabilized for intracellular staining by means of Leucoperm permeabilization kit (Biorad) for MFs/MDSCs and FoxP3 permeabilization kit (Thermofisher) for T cells. Cells were incubated for 30 minutes at room temperature with the antibodies directed against intracellular epitopes. After washing, cells were fixed for 15 minutes at 4°C with 4% paraformaldehyde (PFA). After washing, cells were resuspended in 150 µl of PBS with 1% bovine serum albumin (BSA) and stored at 4°C.

Flow cytometry acquisition was performed on a BD FACSCanto™ II machine (BD Bioscience) within 36 hours from completion of the staining procedure. Results were analyzed using the FlowJo software version 10 (BD Bioscience). Gating strategy for the different T cell, MF and MDSC populations can be found in the Appendix.

Table 2: Extracellular and intracellular antibodies MF and MDSC panel

Target	Fluorophore	Clone	Intra- or extracellular	Amount of antibody (µl)	Company
CD11b	PerCp Cy5.5	M1/70	Extracellular	2	Thermofisher
CD45	APC	104	Extracellular	0,3125	Thermofisher
Ly6G	Fitc	1A8	Extracellular	5	BD Bioscience
Ly6C	APC eFluor780	HK1.4	Extracellular	5	Thermofisher
MHC II (I-A/I-E)	PE-Cy7	M5/144.15.2	Extracellular	0,075	Thermofisher
F4/80	BV421	T45-2342	Extracellular	2	BD Bioscience
CD206	PE	C068C2	Intracellular	2,5	Biolegend

Table 3: Extracellular and intracellular antibodies T cell panel

Target	Fluorophore	Clone	Intra- or extracellular	Amount of antibody (µl)	Company
CD45	APC	104	Extracellular	0,3125	Thermofisher
CD3	APC eFluor780	145-2C11	Extracellular	0,625	Thermofisher
CD4	PerCP Cy5.5	RM4-5	Extracellular	0,625	Thermofisher
CD8	BV421	53-6.7	Extracellular	0,3125	BD Bioscience
FoxP3	AF488	R16715	Intracellular	5	BD Bioscience

3.6. Statistical analysis

Statistical analysis was performed using Graphpad Prism software version 7.0 for Windows (Graphpad Software). For analysis of survival data, a log-rank (Mantel-Cox) test was performed to compare survival between two groups. For immune monitoring experiments, data were tested for normal distribution using a Kolmogorov-Smirnov (if $n \geq 5$) or a Shapiro-Wilk test (if $n < 5$). If data were normally distributed, they were expressed as means and standard deviations and a one-way ANOVA parametric test or an unpaired t test was performed for comparison. If data were not normally distributed, they were expressed as medians and interquartile ranges and a Kruskal-Wallis non-parametric test or a Mann-Whitney test was used for comparison. For all statistical analysis, a p value $< 0,05$ was considered significant.

4. Results

4.1. Single treatments

4.1.1. Survival

Mice receiving RT and TMZ survived significantly longer than untreated tumor bearing controls with a median survival of 33 (p=0,004), 29 (p=0,004) and 25 days, respectively. Anti-PD-1 did not significantly prolong survival compared to control mice (median survival 26 days, p=0,2169). Survival curves for RT, TMZ and anti-PD-1 treated mice are shown in Figure 4.

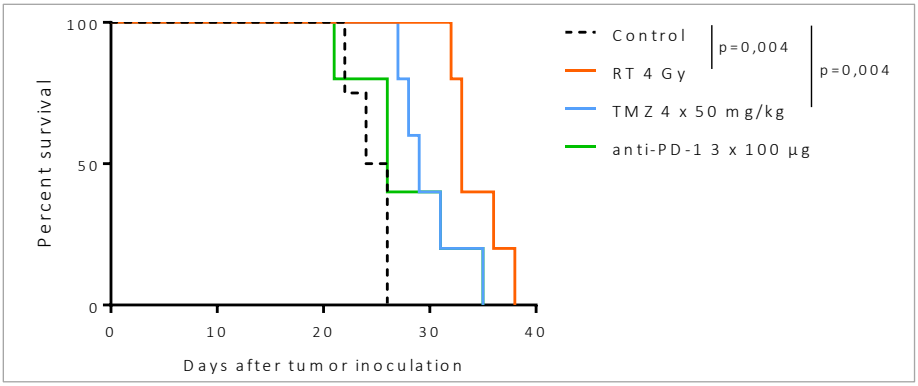


Figure 4: Kaplan-Meier survival curves of mice receiving single treatments (RT, TMZ and anti-PD-1). Both RT and TMZ treatments were able to significantly prolong survival of mice. Statistical analysis was performed with a log-rank (Mantel-Cox) test.

4.1.2. Effect of single treatments on the immune microenvironment

Flow cytometry analysis (FACS) showed no significant differences in any treatment groups compared to controls. However, we found differences on the level of T cells in the tumor microenvironment of mice receiving RT versus TMZ (Figure 5). Irradiated mice had a significantly higher amount of total T cells (p_{adj}= 0,0159), $CD4^+$ T cells (p_{adj}=0,0122) and $CD8^+$ T cells (p_{adj}=0,0078) and a significantly higher $CD8^+/FoxP3^+$ ratio (p_{adj}=0,0134) compared to TMZ treated mice, indicating the contrasting effects of these treatment on the immune biology of GBM. No significant differences were seen for the $FoxP3^+$ regulatory T cell population between any type of treatment and controls; however, RT treated mice showed a reduce amount regulatory T cells even if this did not reach statistical significance. Interestingly, mice treated with anti-PD-1 showed no differences with other treatment groups and control mice.

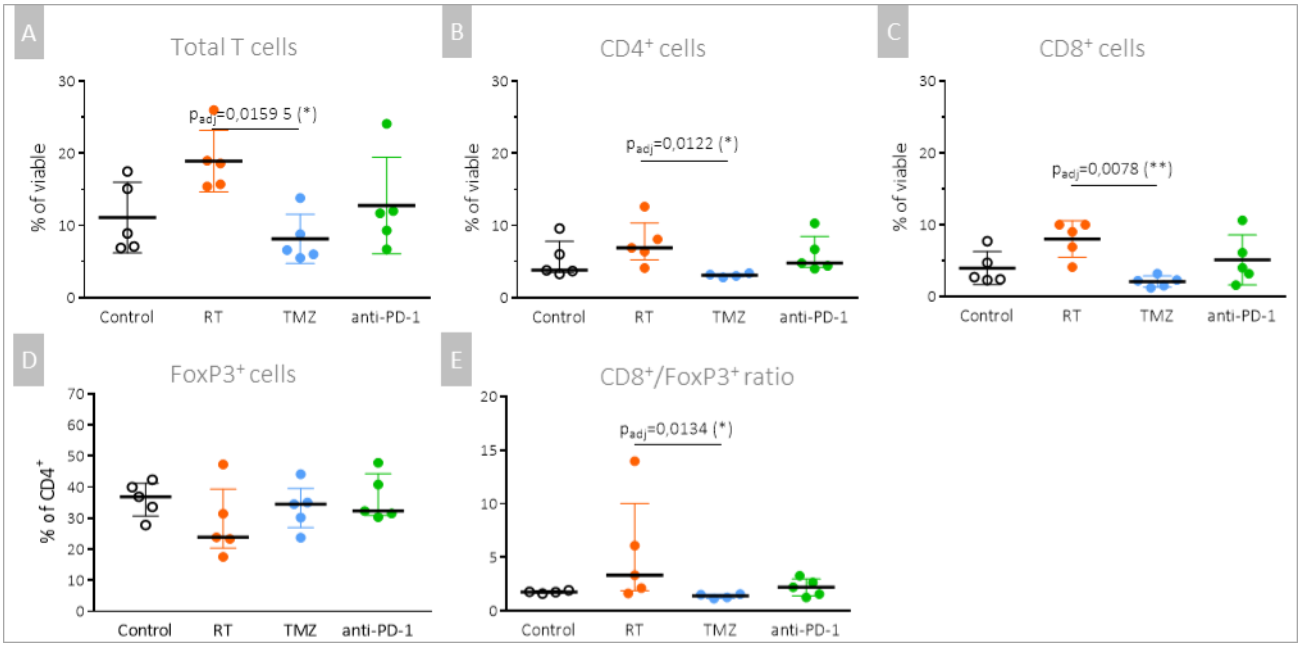


Figure 5: Effects of RT, TMZ and anti-PD-1 on different T cell populations after completion of the therapy (day 19). **A** , total T cells. **B**, $CD4^+$ T cells. **C**, $CD8^+$ T cells. **D**, $FoxP3^+$ regulatory T cells. **E**, $CD8^+/FoxP3^+$ ratio. Statistical analysis was done with a Tukey's multiple comparison test (A, C) or a Kruskal-Wallis test (B, D, E).

Figure 6 shows the effects of single treatments on TAM and MDSC populations. Both RT (p_{adj}=0,0002), TMZ (p_{adj}=0,0167) and anti-PD-1 treatment (p_{adj}=0,0455) were able to significantly reduce the amount of total TAMs compared to untreated controls. RT seemed to have the strongest effect. Both RT and TMZ were able to significantly reduce the M1 fraction of total TAMs (p_{adj} of 0,0010 and 0,0360, respectively); however, only RT was able to significantly reduce the anti-inflammatory M2 fraction (p_{adj}=0,0022).

The total population of MDSCs is decreased in RT (p_{adj}=0,0004), TMZ (p_{adj}=0,0606) and anti-PD-1 treated mice (p_{adj}=0,0905), however only in the RT group this decrease was significant. Both RT and TMZ were able to cause a significant reduction in immune suppressive mMDSCs (p_{adj} of 0,0003 and 0,0439, respectively). Anti-PD-1 didn't cause a significant reduction in mMDSCs but there was a clear tendency towards this effect (p_{adj}=0,0538). In the gMDSC population a significant increase was seen in TMZ treated mice (p_{adj}=0,0021) compared to controls. Further differences were observed between different treatment groups. Radiotherapy treated mice had a lower amount of mMDSCs compared to TMZ and anti-PD-1 treated mice (though not statistically significant). TMZ treated mice had a significantly higher amount of immune suppressive gMDSCs

compared to RT treated mice (p_{adj}=0,0261) and anti-PD-1 treated mice (p_{adj}=0,0098). Even though there was an increase in gMDSCs in TMZ treated mice compared to controls (p=0,0021), it should be noted that the amount of mMDSCs present in the tumor microenvironment was tenfold higher and thus seemingly more relevant in terms of abundance and immune suppressive capacities (145,146).

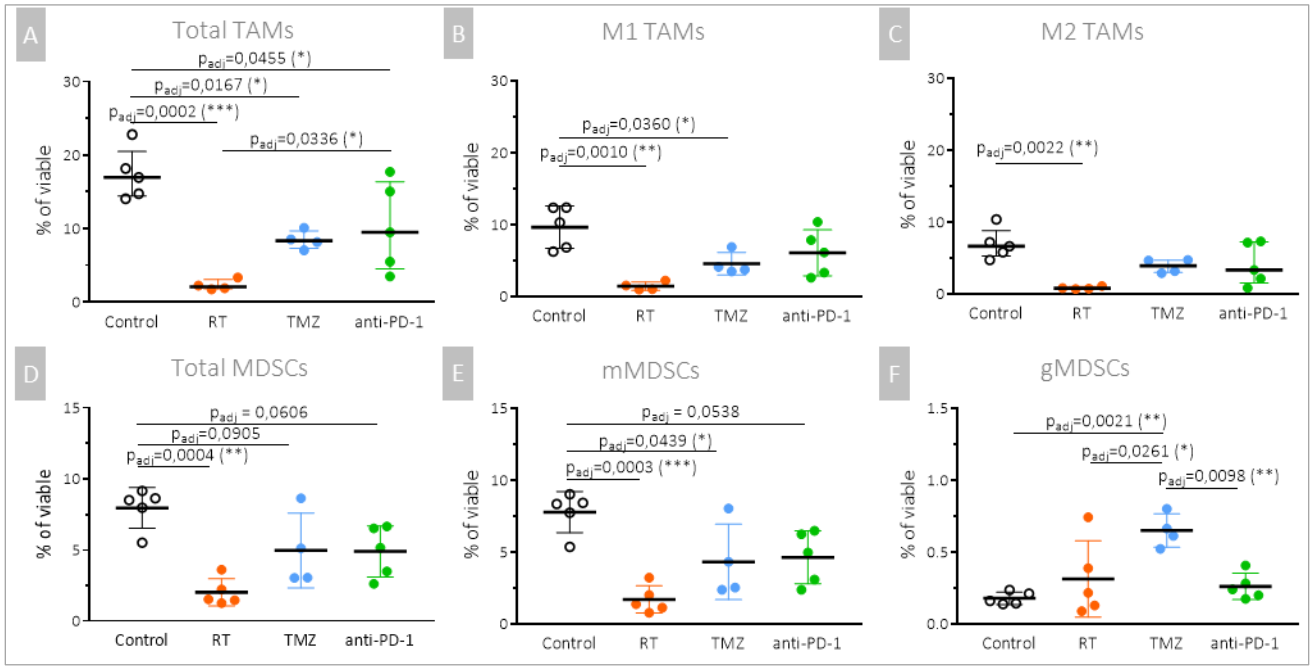


Figure 6: Effects of RT, TMZ and anti-PD-1 on different TAM and MDSC populations after completion of the therapy (day19). A, total TAMs. **B**, M1 TAMs. **C**, M2 TAMs. **D**, total MDSCs. **E**, mMDSCs. **F**, gMDSCs. Statistical analysis was done with a Tukey's multiple comparison test (B, D, E, F) or a Kruskal-Wallis test (A, C).

4.1.3. Responders vs non-responders

As described in Figure 2, the same data set was also analyzed stratifying mice in responders and non-responders independently of the treatment that these mice received. As shown in Figure 7, responding mice had a significantly higher percentage of total T cells (p_{adj}=0,0120) and $CD4^+$ T cells (p_{adj}=0,0477) compared to non-responders. When looking at immune suppressive $FoxP3^+$ regulatory T cells, responders had a significantly lower percentage of this cell population than both control and non-responding mice (p_{adj} value of 0,0227 and 0,0006, respectively).

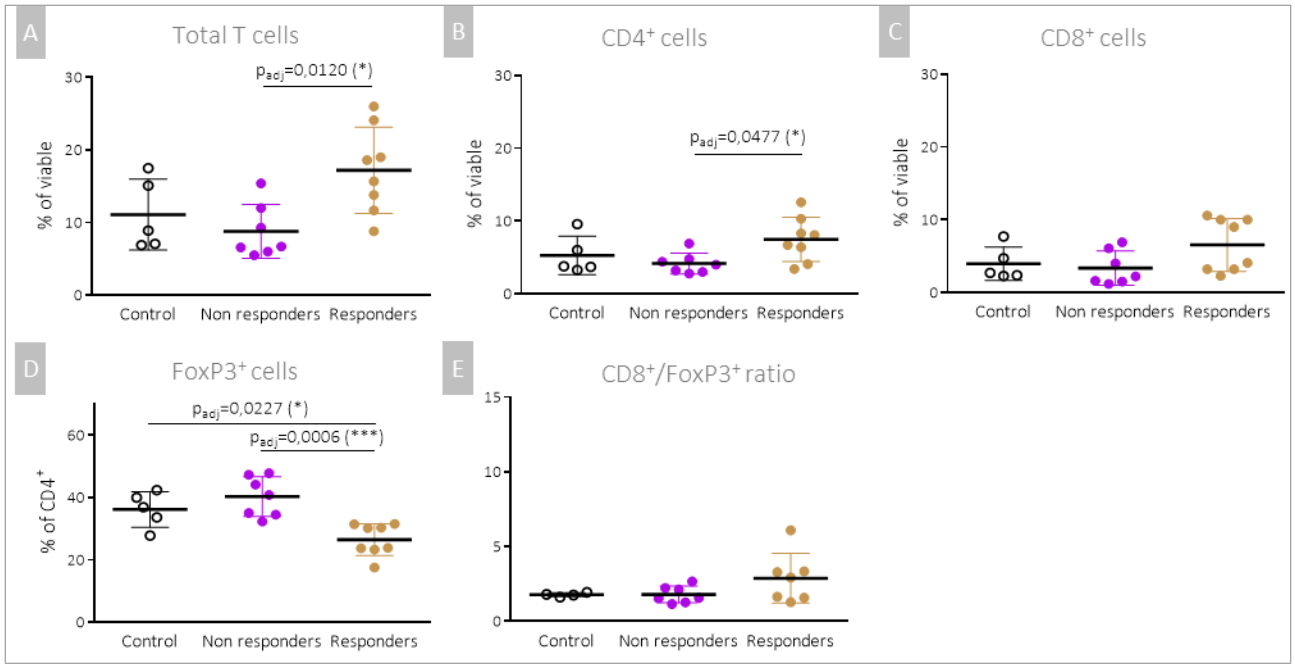


Figure 7: Differences between responders and non-responders on different T cell populations after completion of their therapy (day 19). A , total T cells. **B**, $CD4^+$ T cells**. C**, $CD8^+$ T cells. **D**, $FoxP3^+$ regulatory T cells. **E**, $CD8^+/FoxP3^+$ ratio. Statistical analysis was done with a Tukey's multiple comparison test.

It was also observed that responders had a significantly lower amount of total TAMs (p_{adj}=0,0016), M1 TAMs (p_{adj}=0,0005) and M2 TAMs (p_{adj}=0,0029) compared to control mice while non-responders showed only a decrease in the amount of M1 TAMs compared to control mice (p_{adj}=0,0484). No significant effect was seen on the anti-inflammatory M2 fraction (Figure 8). The only difference between responders and non-responders was found in the M2 TAM population where a significant decrease was seen in the responding mice (p_{adj}=0,0432). Furthermore, both responders and non-responders showed a significant decrease in the total MDSC population (p_{adj} values 0,0013 and 0,0270, respectively) and in mMDSCs (p_{adj} value of 0,0009 and 0,0222, respectively) compared to controls. Although both responders and non-responders showed this decrease, it was much stronger in the responding tumors. mMDSCs accounted for 7,78% of viable cells in control mice, 4,39% in non-responders and 2,61% responders. Similar trend was seen for total MDSCs. For gMDSCs no significant differences were seen between different groups. Results for MF and MDSC populations are shown in Figure 8.

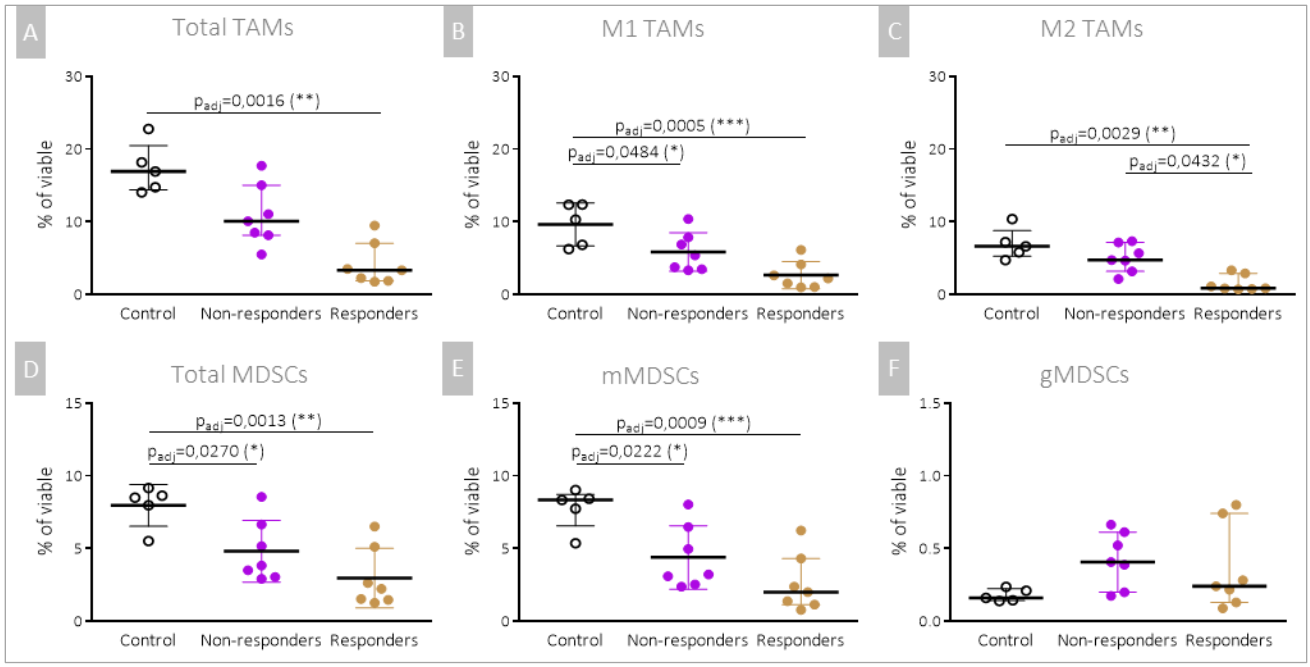


Figure 8: Differences between responders and non-responders on TAM and MDSC populations after completion of their therapy (day 19). **A**, total TAMs. **B**, M1 TAMs. **C**, M2 TAMs. **D**, total MDSCs. **E**, mMDSCs. **F**, gMDSCs. Statistical analysis was done with a Tukey's multiple comparison test (B, D, E) or a Kruskal-Wallis test (A, C, F).

4.2. Combination treatments

In this part of the experiment, two main subanalysis were made of combination schedules shown in Figure 2. In a first subanalysis we compared both survival and immunomonitoring data of RT, RT – TMZ and RT – TMZ – anti-PD-1 (implying anti-PD-1 treatment after completion of RT – TMZ) treated mice in order to assess the effects of the current human standard of care (RT – TMZ) in our model and the impact of anti-PD-1 in the schedule normally used in ongoing clinical trials (RT – TMZ- anti-PD-1). In a second subanalysis of the same experiment, a comparison was made between RT – TMZ and all triple combination schedules possible using RT, TMZ and anti-PD-1. The aim was to investigate if the moment of anti-PD-1 administration could influence the outcome.

4.2.1. Survival

Survival comparison of mice treated with RT, RT – TMZ and RT – TMZ – anti-PD-1 (first subanalysis) is shown in Figure 9. The addition of TMZ to RT significantly prolonged survival of mice with a median survival of 39 days for RT – TMZ compared to 33 days for RT alone (p=0,0142). This is in accordance to what is observed in the clinical situation, where RT – TMZ represents the standard of care for GBM patients. However, adding anti-PD-1 to the end of the treatment schedule (RT – TMZ – anti-PD-1) did not induce a significant survival improvement, only a trend towards prolongation of survival compared to mice receiving RT – TMZ (median survival 43 vs 39 days, p=0,0842). One long term survivor was observed.

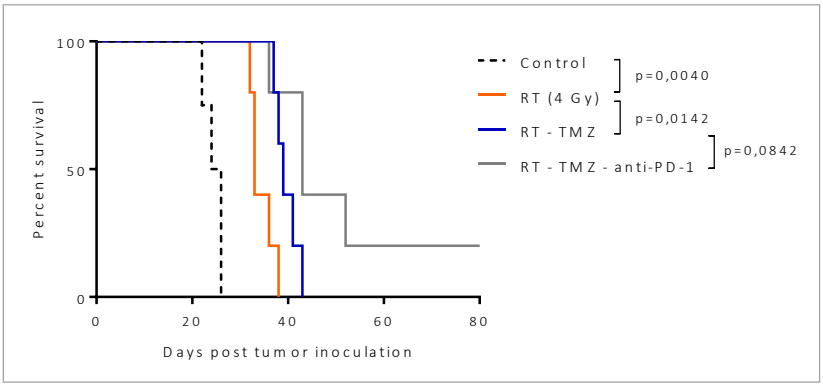


Figure 9: Kaplan-Meier survival curves for RT, RT – TMZ and RT – TMZ – anti-PD-1 treated mice. Adding TMZ to the treatment schedule significantly prolongs survival of mice (p=0,0142) to 39 days compared to 33 days in the RT group. In the RT – TMZ – anti-PD-1 treatment group, median survival is 43 days and contains one long term survivor (p=0,0842).

In the second subanalysis of this experiment (Figure 10), we evaluated if survival could be improved by changing the order of the three components: RT, TMZ and anti-PD-1 (Figure 2). However, no schedule was superior compared to RT – TMZ alone or the previously evaluated RT – TMZ – anti-PD-1 schedule (administering anti-PD-1 after RT – TMZ treatment).

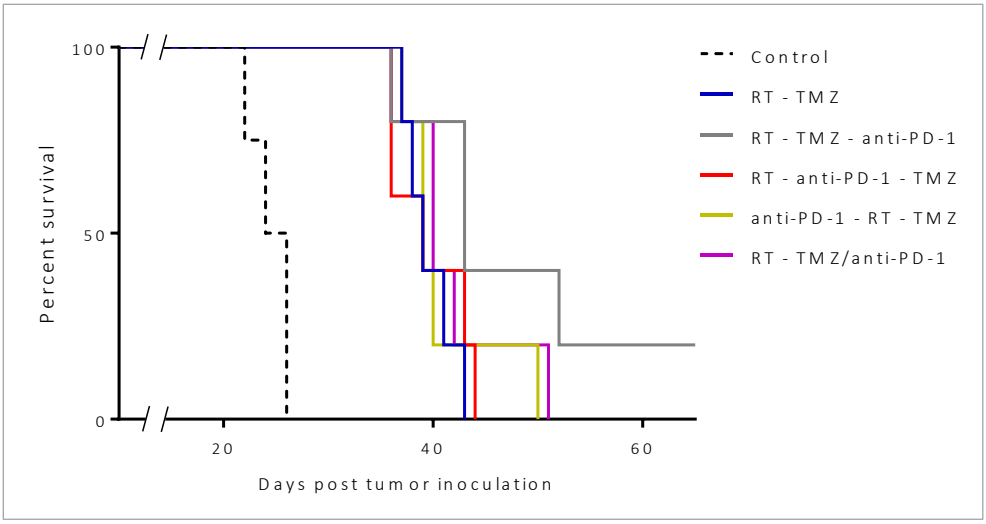


Figure 10: Kaplan-Meier survival curves of mice treated with different combination treatment schedules including RT, TMZ and anti-PD-1. No differences are seen between different combination schedules compared to the already existing RT-TMZ standard treatment. One long term survivor was seen in the RT – TMZ – anti-PD-1 group. Statistical analysis was performed with a log-rank (Mantel-Cox) test.

4.2.2. Effect of combination treatments on GBM immune microenvironment

For this part, the same subdivision was maintained as used in 4.2.1. The analysis of tumor-infiltrating immune cells of mice treated with RT, RT – TMZ and RT – TMZ – anti-PD-1 is shown in Figure 11 (T cells) and 12 (TAMs and MDSCs). Mice receiving the standard treatment (RT – TMZ) showed a significant reduction in $CD8^+$ T cells (p_{adj}=0,0145) and a trend toward a decrease in $CD8^+/FoxP3^+$ ratio (p_{adj}=0,1302) compared to RT treated mice. Similarly to RT – TMZ, RT – TMZ – anti-PD-1 showed a significant reduction in $CD8^+$ T cells (p=0,0055) and in addition a clear trend towards a decrease in total T cells (p=0,0661), $CD4^+$ T cells (p=0,0754) and immune suppressive $FoxP3^+$ T cells (p=0,0669) compared to the single treatment of RT. Furthermore, the $CD8^+/FoxP3^+$ ratio, a measurement of the activation status of the immune system, showed a clear tendency towards an increase in the RT – TMZ – anti-PD-1 group compared to RT – TMZ (p=0,0973), partially rescuing the negative effect of RT – TMZ on this ratio.

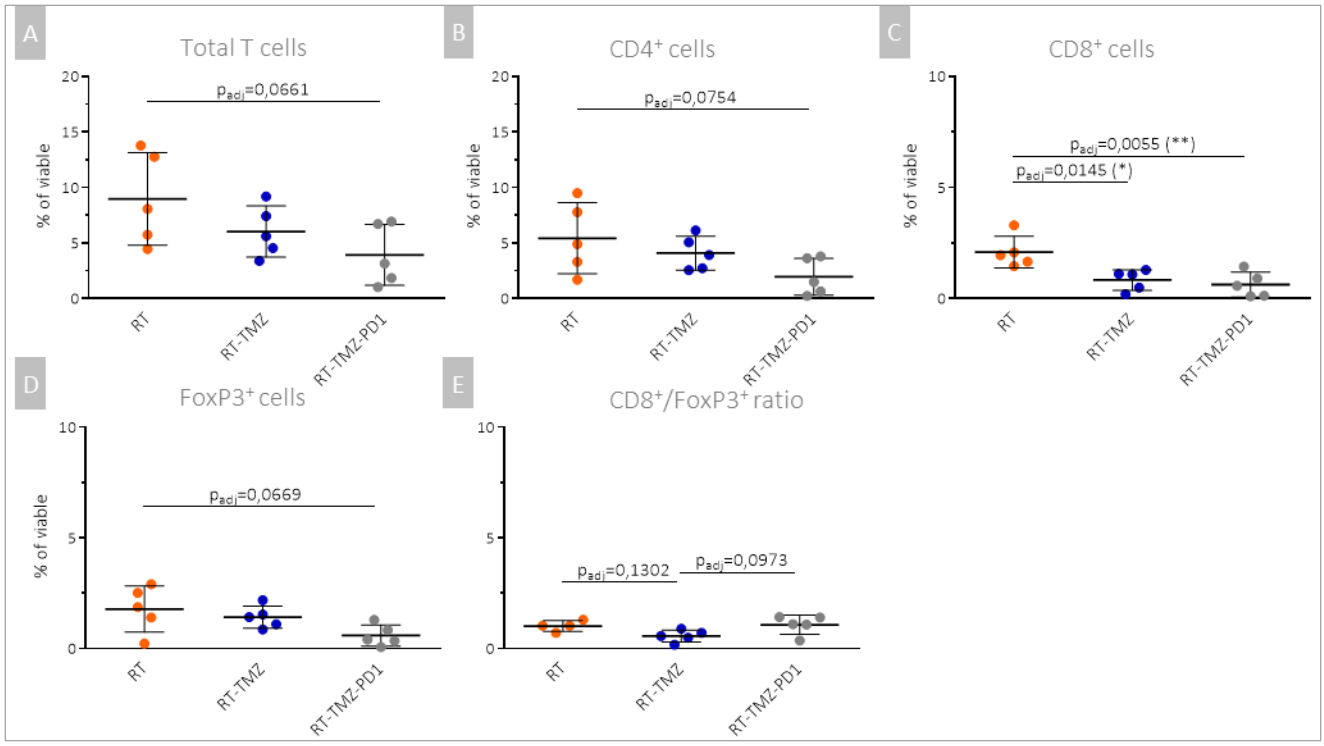


Figure 11: Effects of RT, TMZ and anti-PD-1 in combination schedules (RT, RT – TMZ, RT – TMZ – anti-PD-1) on T cell populations after completion of the therapy (day 28). **A** , total T cells. **B**, $CD4^+$ T cells. **C**, $CD8^+$ T cells. **D**, $FoxP3^+$ regulatory T cells. **E**, $CD8^+/FoxP3^+$ ratio. Statistical analysis was performed with a Tukey's multiple comparison test.

Concerning MFs, the RT – TMZ – anti-PD-1 induced a general reduction of the whole cell population compared to RT and RT - TMZ, although this is not supported by statistical evidence. However, in the RT – TMZ – anti-PD-1 treated mice a trend was seen towards a reduction of M2 TAMs compared to the standard clinical treatment (RT – TMZ). Furthermore, this triple combination induced a non-significant reduction in both total MDSCs and mMDSCs which was not present in the gMDSC population.

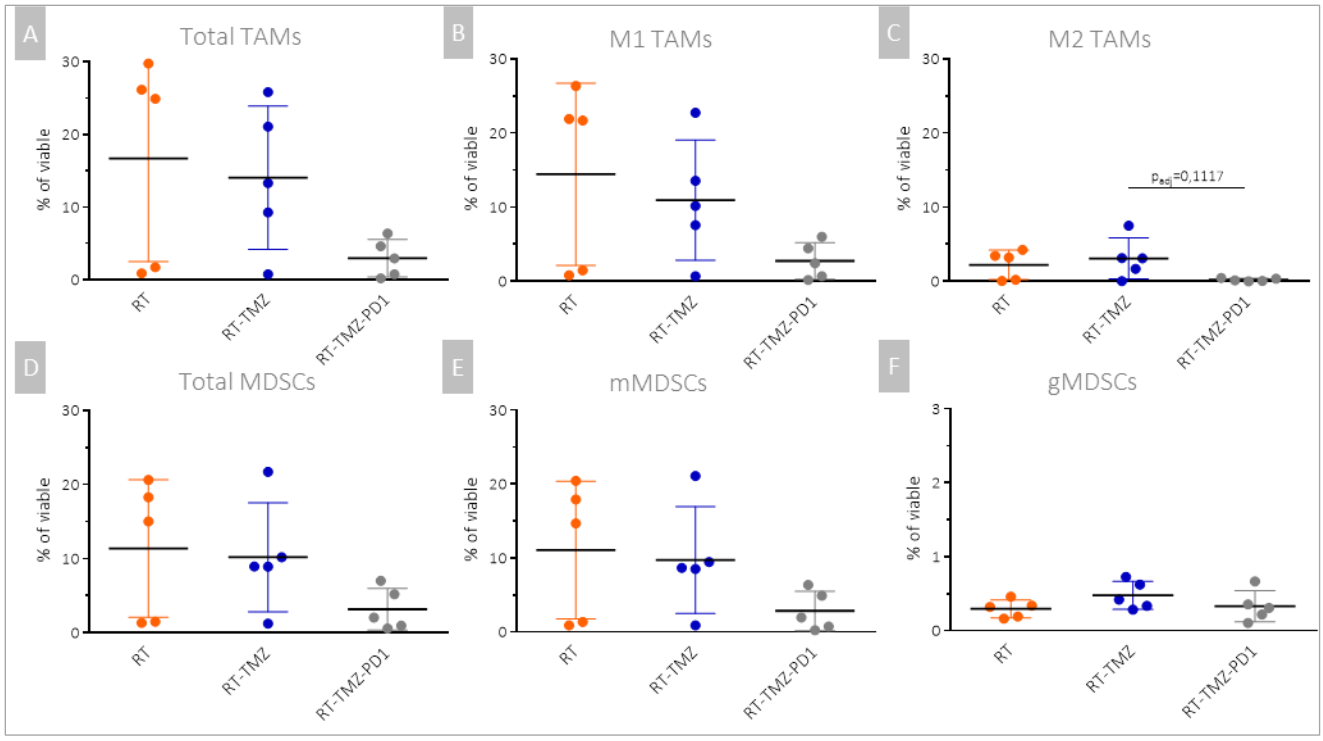


Figure 12: Effects of RT, TMZ and anti-PD-1 in combination schedules (RT, RT – TMZ, RT – TMZ – anti-PD-1) on TAM and MDSC populations after completion of the therapy (day 28). **A**, total TAMs. **B**, M1 TAMs. **C**, M2 TAMs. **D**, total MDSCs. **E**, mMDSCs. **F**, gMDSCs. Statistical analysis was performed with a Tukey's multiple comparison test.

The analysis of tumor-infiltrating immune cells of mice treated with RT – TMZ and the four triple combination therapies (second subanalysis) of the experiment is shown in Figure 13 (T cells) and 14 (TAMs and MDSCs). However, no significant differences were observed between triple combinations and RT – TMZ treated mice and among the four different triple combination schedules.

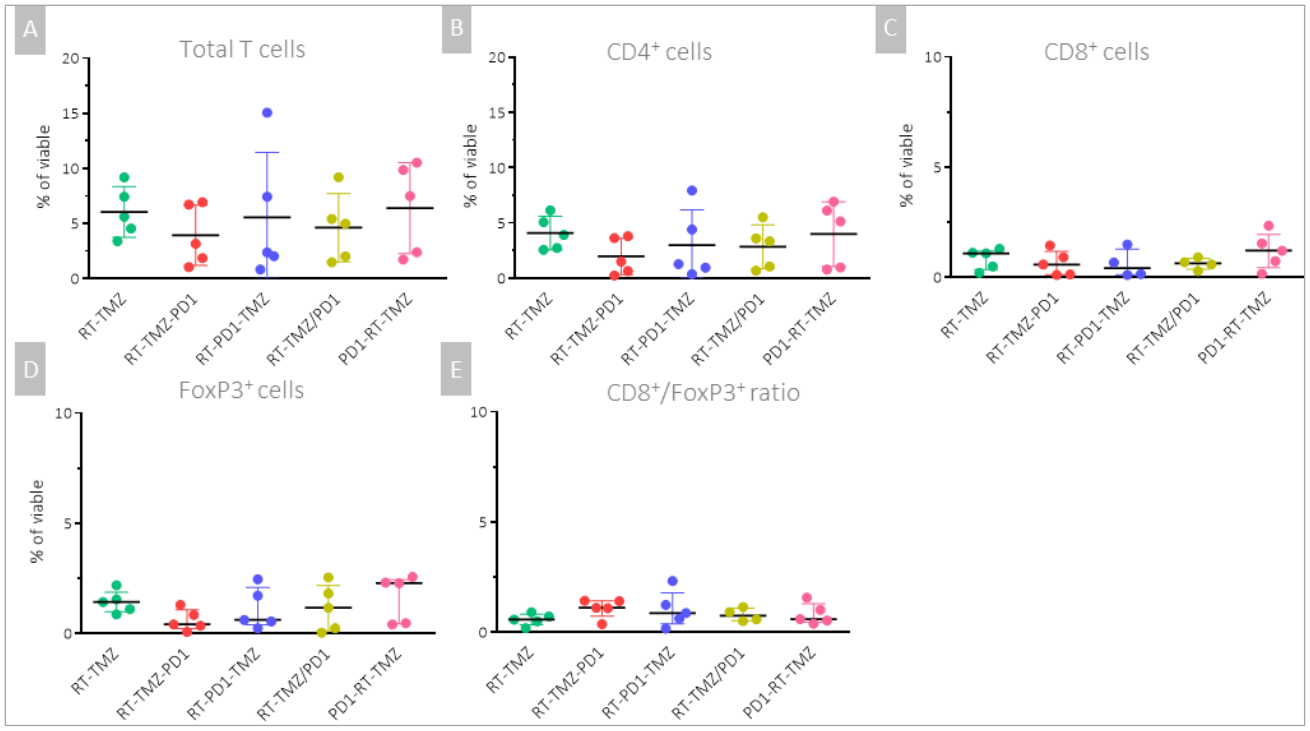


Figure 13: Effects of RT, TMZ and anti-PD-1 in different combination schedules on T cell populations after completion of the therapy (day 28). **A** , total T cells. **B**, $CD4^+$ T cells. **C**, $CD8^+$ T cells. **D**, $FoxP3^+$ regulatory T cells. **E**, $CD8^+/FoxP3^+$ ratio. Statistical analysis was performed with a Tukey's multiple comparison test (A, B, C, E) or a Kruskal-Wallis test (D).

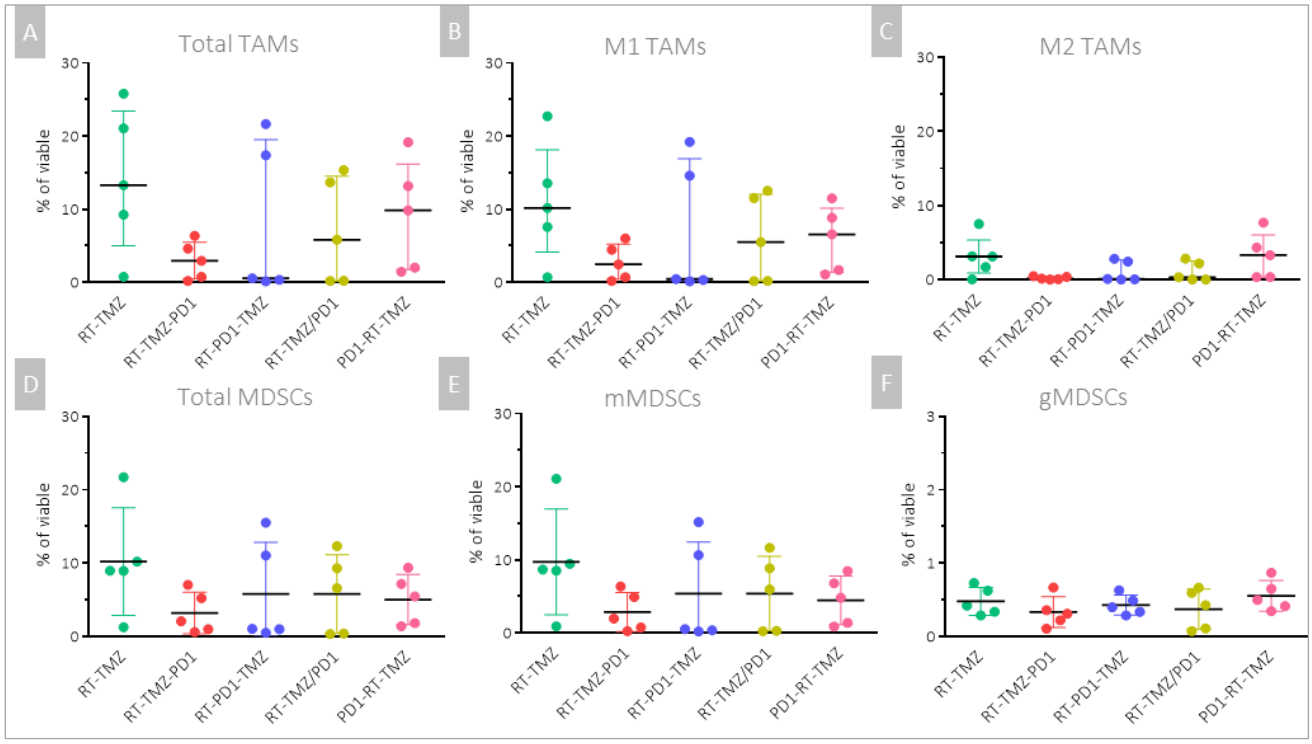


Figure 14: Effects of RT, TMZ and anti-PD-1 in different combination schedules on TAM and MDSC populations after completion of the therapy (day 28). **A**, total TAMs. **B**, M1 TAMs. **C**, M2 TAMs. **D**, total MDSCs. **E**, mMDSCs. **F**, gMDSCs. Statistical analysis was performed with a Tukey's multiple comparison test (D, E, F) or a Kruskal-Wallis test (A, B, C).

4.2.3. Responders vs non-responders

Contrary to the results obtained for the single treatments, the brain tumor microenvironment of responders showed a significant decrease in all immune cell types compared to non-responder mice. Results for T cell populations are shown in Figure 15. A reduction was seen in total T cells (p=0,0080), $CD4^+$ T cells (p=0,0116) and $CD8^+$ T cells (p=0,0077) and $FoxP3^+$ T cells (p<0,0001). However, the $CD8^+/FoxP3^+$ ratio was not affected in the responder mice.

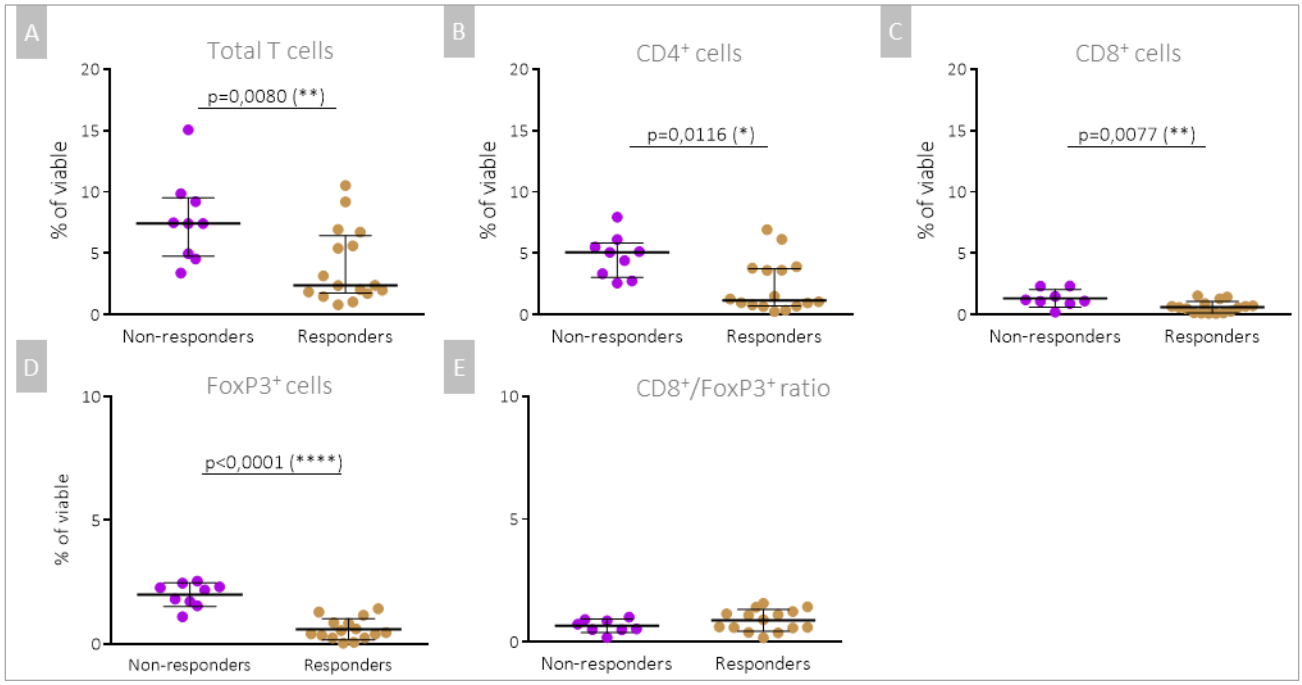


Figure 15: Differences in T cell populations between responders and non-responders after completion of their combination therapy (day 28). **A** , total T cells. **B**, $CD4^+$ T cells. **C**, $CD8^+$ T cells. **D**, $FoxP3^+$ regulatory T cells. **E**, $CD8^+/FoxP3^+$ ratio. Statistical analysis was performed with a Mann Whitney test (A, B) or an unpaired t test (C, D, E).

Likewise, a significant reduction was seen in the amount of total TAMs (p<0,0001), M1 (p<0,0001) and M2 TAMs (p<0,0001) in responding tumors compared to non-responders, which is shown in Figure 16. The same trend was seen for all MDSC populations where both total MDSCs (p<0,0001), mMDSCs (p<0,0001) and gMDSCs (p=0,0257) were significantly decreased in the brain tumor microenvironment of responders.

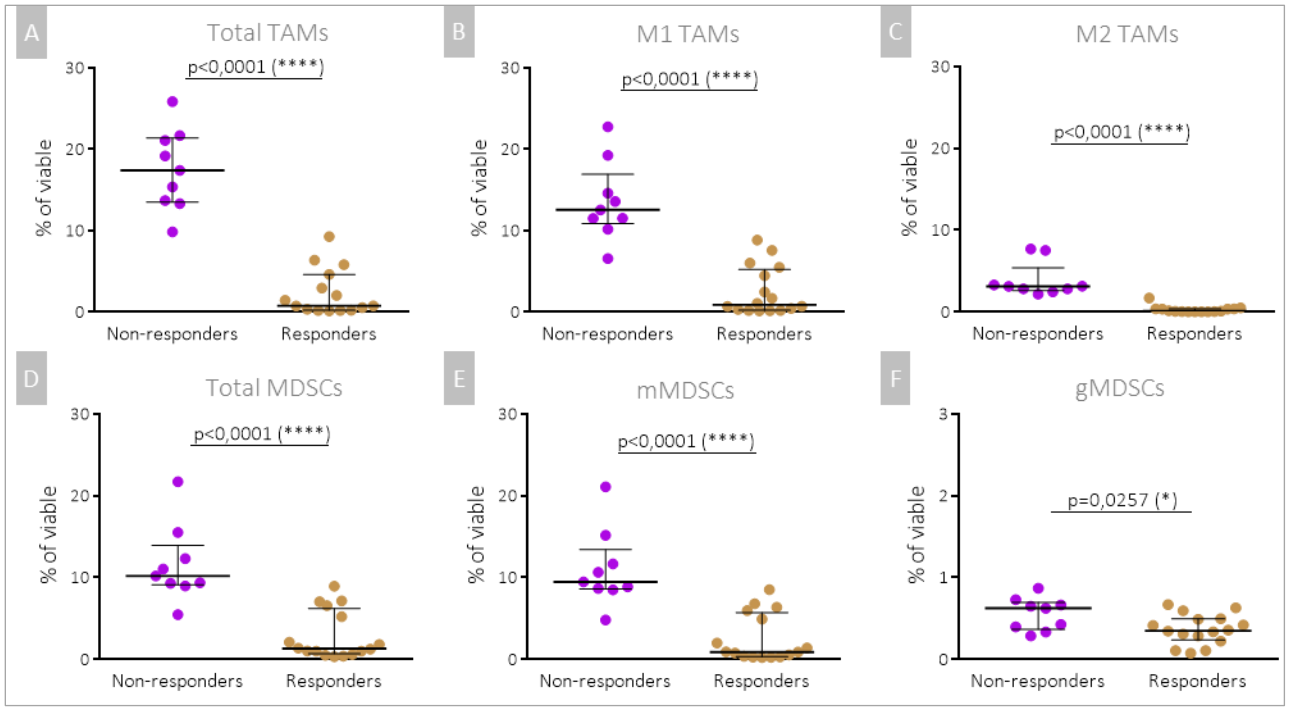


Figure 16: Differences in TAM and MDSC populations between responders and non-responders after completion of their therapy (day 28). A, total TAMs. **B**, M1 TAMs. **C**, M2 TAMs. **D**, total MDSCs. **E**, mMDSCs. **F**, gMDSCs. Statistical analysis was performed with a Mann Whitney test (A, B, C, D, E) or an unpaired t test (F).

5. Discussion and conclusion

5.1. Clinical relevance of the project

Glioblastoma is the most common primary malignant brain tumor with a median survival of only 14,6 months despite the current standard of care, including surgical resection followed by radio- and chemotherapy. Despite several attempts to develop new and improved treatment strategies, including immunotherapy, prognosis remains poor. We believe that one of the main reasons why immunotherapy is still not succeeding in the clinic is the fact that no sufficient data were available on the immune-changing effects of RT, TMZ and immunotherapeutic strategies on the microenvironment of GBM, neither in a preclinical nor clinical setting. Since other studies already provided some hints about the possible immune effect of conventional treatments (RT and TMZ) on a more systemic level (126,132), the information about the specific effects of these treatments on tumor-infiltrating immune cells of GBM is crucial in trying to understand how immunotherapy needs to be administered (in which order, at which time point), in order to synergize with conventional treatments and improve patients' prognosis. Complete and extensive understanding of this aspect is lacking in current literature. This is probably why immune checkpoint inhibitors such as anti-PD-1, which were proven to be effective in mice, fail to show survival benefit in recent clinical trials (143). In this research, we aimed at filling this existing knowledge gap and at providing such essential information.

5.2. Discussion of obtained results

We first demonstrated that single administrations of both RT and TMZ were able to significantly prolong survival of mice compared to controls (in line with the clinical reality), however, anti-PD-1 (when administered as a single treatment) was not. Contrary to our results, others have been able to cure 55% of animals with anti-PD-1 administered as a single treatment (147). However this was shown in the GL261 instead of the CT-2A mouse model and anti-PD-1 was given in a higher dosage. Nevertheless, we believe that the effect of anti-PD-1 in our study is more

clinically relevant in view of the available clinical literature in which anti-PD-1 is shown to have (at best) a limited impact on patients survival (143).

To assess the effects of RT, TMZ and anti-PD-1 on the immune-biology of GBM, we evaluated their effects on the brain tumor immune microenvironment. Interestingly, RT and TMZ had contrasting effects on different T cell populations (total T cells, $CD4^+$ T cells and $CD8^+$ T cells). While RT had a rather activating effect on adaptive the immune system, TMZ was clearly immune suppressive. In general, the immune activating effects of RT and immune suppressive effects of TMZ on the immune system of tumor patients have been partially described before by others (126,132); however, this is the first time that this analysis is performed on the tumor infiltrating immune cells of GBM and with such degree of detail. The contrasting effect of RT and TMZ is especially noteworthy since both therapies are given together in the clinic as part of the standard of care and TMZ seems to partially neutralize the immune activating effect of RT, which was indeed confirmed in our combinatorial setting in a later experiment. These effects were in accordance to survival analysis: RT with the strongest immune activating effect, was correlated to the most beneficial effect on survival in mice. TMZ was able to induce positive effects on survival, though probably more because of its direct cytotoxic effect on tumor cells rather than through immune stimulation. Another remarkable result is the fact that anti-PD-1, the only immunotherapy in a strict sense, had the least effect on the infiltrating immune cell populations of GBM when given as a single treatment compared to the conventional therapies such as RT and TMZ. These results correlated again to survival data where no significant survival benefit was seen in the anti-PD-1 treated group compared to untreated control mice.

Our next focus consisted in the evaluation of the combined effect of the standard of care (RT-TMZ) with and without anti-PD-1 in order to shed some lights on its effects on the tumor-infiltrating immune cells in mice. This is crucial information when trying to understand why anti-PD-1 treatment failed to significantly improve prognosis of GBM patients so far. Our results clearly showed that adding TMZ to the RT significantly reduced the amount of $CD8^+$ T cells and caused trend towards a reduction in the $CD8^+/FoxP3^+$ ratio, and thus a reduced activation status of the immune system, compared to RT treated mice. Others have reported similar findings on all T cell populations in circulating blood of GBM patients after administration of RT – TMZ (137,148). On

the other hand, evidence for the opposite effect of TMZ has also been described. Sampson et al. (134) reported a greater tumor-specific immune response in combination with an EGFRvIII-targeted peptide vaccine when TMZ induced lymphopenia was more pronounced, an effect that can be attributed to an increased antigen-specific T cell expansion during the recovery phase from this lymphopenia (149). In this view, our results are important since they show that what is observed in circulating immune cells may not always recapitulate the modifications present in the tumor microenvironment.

Furthermore, adding anti-PD-1 to the schedule partially rescued the detrimental effect of TMZ on the $CD8^+/FoxP3^+$ ratio. However, this effect of anti-PD-1 was not sufficient to significantly improve prognosis of RT – TMZ – anti-PD-1 treated mice as observed in our survival experiments. Nevertheless, although no survival benefit was observed in mice receiving anti-PD-1 as a single treatment, when adding it to the current standard of care specifically at the end of the schedule (RT – TMZ – anti-PD-1), we were able to show a trend towards prolongation of survival compared to RT – TMZ alone. Of note, this treatment group was also the only one where a long-term survivor was observed. As discussed before, the addition of anti-PD-1 in this particular schedule was able to partially revert the reduction in the $CD8^+/FoxP3^+$ ratio caused by TMZ in RT – TMZ treated mice.

In addition, important effects were observed concerning the innate immune system. All single treatments were able to reduce the total amount of TAMs, however only RT was able to significantly reduce the anti-inflammatory M2 fraction. In addition to the effects on the innate immune system seen in the monotherapy experiments, in combinatorial experiments RT – TMZ – anti-PD-1 also showed a trend towards a reduction in M2 TAMs, compared to RT – TMZ treated mice. TAMs of the M2 phenotype play an important role in processes such as angiogenesis and are thus promoting tumorigenesis. However, these effects have not directly been assessed in our study. Others have described the role of TAMs in angiogenesis in an *in vitro* CT-2A setting and have demonstrated that indeed the M2 fraction is promoting the process of angiogenesis in this GBM model *in vitro* (150). Additionally, the process of TAM polarization towards an M1 or M2 phenotype is characterized by a strong plasticity (151). This indicates that possibly these cell populations should be looked at as a whole. Others have reported that in GBM the M1 and M2 classification of TAMs is oversimplified and that is should be seen as a spectrum of different TAM

phenotypes (152). An attempt to convert an M2 phenotype more towards the M1 side of the spectrum can potentially be a solution to overcome the immune suppressive effects of these M2 TAMs.

In our experiments, RT was also able to significantly reduce the amount of total MDSCs and mMDSCs, however this trend was also present in TMZ and anti-PD-1 treated mice. The role of mMDSCs, and in lesser extent the role of gMDSCs, in the mouse brain tumor microenvironment of GBM was another important finding in our study concerning the innate immune system. It should be noted that in our model mMDSCs were tenfold more abundant than gMDSCs, indicating that the mMDSCs might be more relevant in our experimental setting. Furthermore, it is generally accepted that the mMDSC subpopulation has a more potent effect in terms of immune suppressive capacities (145,146). Strikingly, the clinical situation is completely opposite compared to what was demonstrated in our model. Gielen et al. reported a predominant presence of $CD15^+$ MDSCs in glioma tissue derived from patients which correlates to the $Ly6C^{low}Ly6G^+$ gMDSC phenotype in mice (153). This discrepancy reminds us that a translational and probably also a nomenclature gap exists between murine and clinical setting in GBM. It would be extremely useful for future studies to address more in detail the immune suppressive capacities of both mMDSCs and gMDSCs specifically in GBM to provide more information about their individual roles. For this purpose, in vitro studies or MDSC knockout models might be of use. Nonetheless, we believe that the decrease in total MDSCs, independently of their phenotype, needs to be considered for its beneficial effect in counteracting the immune suppressive environment of HGGs.

The role of the innate immune system in GBM has been assessed by others and correlates strongly with our results. Administering an anti-CCL2 monoclonal antibody, thereby blocking the recruitment of TAMs and MDSCs to the tumor microenvironment, in combination with TMZ was associated with a significant survival benefit and a concomitant decrease in both TAMs and MDSCs in the tumor microenvironment of a GL261 murine glioma model (154).

Taking into account its effects on both the adaptive ($CD8^+$/$FoxP3^+$ ratio) and the innate (M2 TAMs and MDSCs) immune system, the RT – TMZ – anti-PD-1 combinatorial regimen certainly holds promise, however, this treatment schedule has been recently tested in a phase III clinical trial (NCT02017717). Preliminary results of this trial indicated that Nivolumab (an anti-PD-1

monoclonal antibody) when added at the end of the current standard of care, had no significant effect on survival in recurrent GBM patients. Once again, this highlights the strong need to further improve the treatment schedules including checkpoint inhibitors for GBM used in clinical trials. In our combination schedules, we have shown that changing the moment of administration of anti-PD-1 does not change survival nor the immunobiology of the tumor in mice.

Lastly we demonstrated the clear difference in immunological profile between mice responding or not to treatments, as they were divided based on the increase in tumor volume before and after treatment.

In the single-treatments setting, responders showed a clear shift towards a reduced immune suppression and increased immune stimulation in terms of T cell populations. Responders also had a higher $CD8^+/FoxP3^+$ ratio clearly indicating their stronger immune activation. These results prove the relevance of the immune system in the treatment of GBM and may partially explain the difference among patient groups responding or not to the same treatments. Furthermore, modifications in the TAM and MDSC populations indicate also that the innate immune system plays a role in the tumor development as the adaptive immune system. In our single-treatment experiments, mMDSCs showed a decrease in all treatments, however this decrease was much more prominent in responders compared to non-responders, proving that a less immune suppressive environment was beneficial in terms of reduction of the tumor volume.

When we performed the same analysis of responders and non-responders in the combination-treatment settings, responders showed a general reduction in all immune cell populations (T cells, TAMs and MDSCs) and the immune activation shift in terms of $CD8^+/FoxP3^+$ ratio was also not present (in contrast to what was seen in monotherapy). At present, we have no clear explanation for this. However, we believe that this could be partially related to the fact that the stratification between responders and non-responders in combination experiments was based on the tumor volume data obtained from the monotherapy experiments, since control mice did not survive long enough to be used for this purpose in the experiments with combination treatments. Because of this reason, the division between responders and non-responders was more artificial in the combination experiments compared to single treatment experiments.

5.3. Limitations and strengths

Our study suffered of a few limitations. The first limitation is related to sample size (n=5 in each group) of the experiments. In some cases we were not able to demonstrate a significant difference between different groups although we observed a clear tendency towards a certain effect. Including more mice might have provided additional statistical strength to this study. However, practical limitations due to the difficulties to work with more than a certain number of mice at the same time hampered us to include more animals in the treatments groups, especially in flow-cytometry experiments. The second limitation was the absence of activation markers for different immune cell populations in our flow cytometry analysis. Including this information could have provided additional knowledge to the abundancy data that we have obtained so far for different cell types. For instance, assessing the activation status of the tumor-infiltrating $CD8^+$ cytotoxic T cells could have given interesting information concerning their actual actuation status vs exhaustion. A possible way to explore the activation of $CD8^+$ T cells is via analysis of cytokine production or expression of surface activation markers. The third limitation was the lack of steroid treatment. Corticosteroids are often used peri-operatively to control brain edema caused by the tumor and the surgical procedure. These drugs have additional and strong immune modulating effects that were not analyzed in this research project.

Additionally, this project also possesses some strengths. Our model has a much closer resemblance to the clinical situation since we included MRI imaging and a focal irradiation strategy. These are two rather advanced techniques to be used in a murine setting and strongly increase the translational impact of our study compared to other studies. Furthermore, the readout of effects on both the adaptive and innate immune system gives an additional advantage since our results clearly indicate that both are relevant in relation to tumor development.

5.4. Conclusion and future implications

In immunotherapy clinical trials so far conducted, one possible reason for the failure of the treatments was the lack of fundamental data regarding the immune biology of GBM. This essential data would have been necessary to incorporate immunotherapies into the already existing treatment schedule and to obtain a synergistic effect.

In the present study, we have provided sufficient evidence on the immunological effects on both the adaptive and innate immune system of RT, TMZ and anti-PD-1 given as single treatments and in several combination schedules. We analyzed the immune cells infiltrating the tumor, which most likely are the more relevant in this context given the peculiar aspects of the brain immune system. We demonstrated that RT has powerful immune activation properties, while TMZ exerts the opposite effect. Furthermore, TMZ immune suppression was particularly strong and was evident in all the combination schedules. Interestingly, the addition of anti-PD-1 was not sufficient to revert this condition: this could partially explain why clinical trials in which patients were treated with anti-PD-1 after the standard chemotherapeutic regimen failed to prove a survival benefit. Whether the same effects on tumor-infiltrating immune cells are present in GBM patients, remains an open question. A possible next step might be to perform immune monitoring in ongoing immunotherapeutic studies. However, difficulties in accessing human brain tumor samples (via biopsy or surgical removal of the tumor) make such a clinical study particularly difficult.

Several immunotherapies are already available or are emerging in the field of GBM. Combinations including immune checkpoint inhibitors, DC vaccines and oncolytic viral therapy, as well as cell therapies, could potentially produce an impact on the survival of GBM patients. However, the impact of such strategies on the immune biology of the tumor microenvironment needs to be analyzed in order to provide a more synergistic and effective therapy.

Samenvatting

1. Inleiding

Glioblastoma (GBM), een graad IV astrocytoma, is de meest voorkomende kwaadaardige hersentumor met een mediane overleving van 14,6 maanden (107). De huidige behandelingsstrategie bestaande uit chirurgische resectie gevolgd door radiotherapie (RT) en een chemotherapeuticum, namelijk Temozolomide (TMZ), is onvoldoende om voor deze patiënten een betere prognose te voorzien.

Eén van de redenen waarom de prognose van deze patiënten zodanig beperkt is, is de invloed van een sterke immunosuppressieve omgeving in de nabijheid van de tumor veroorzaakt door de aanwezigheid van verschillende celtypes, onder andere macrofagen (MF) van het M2 type, myeloïde-afgeleide suppressor cellen (MDSC), regulatorische T cellen (T_{reg}) en toegenomen activatie van de immuun checkpoints (interactie van geprogrammeerde dood ligand 1 (PD-L1) met zijn receptor PD-1 en cytotoxisch T lymfocyt antigen 4 (CTLA-4)) (52,66,70,89,102). Al deze factoren maken dat cellen van het adaptieve immuunsysteem, de $CD4^+$ en $CD8^+$ T cellen, niet voldoende efficiënt geactiveerd kunnen worden om de tumorcellen doelgericht met behulp van het immuunsysteem te elimineren. Het gericht aanpakken van deze onderdrukking van het immuunsysteem, kan nieuwe perspectieven openen in de behandelingsstrategie van GBM patiënten.

Experimentele behandelingen, onder andere anti-PD-1 immuuntherapie, hebben klinisch nog geen verbetering kunnen bereiken op dit vlak. Een belangrijke verklaring hiervoor zou kunnen zijn dat immune checkpoint inhibitors worden toegepast zonder gegevens over de immunologische veranderingen die de standaard therapie reeds veroorzaakt. Het is geweten dat TMZ een toxisch effect heeft op immuun cellen en dat RT door middel van immunogene cel dood het immuun systeem gericht kan stimuleren tegen de tumorcellen (126,132). Het doel van dit onderzoek was dan ook om de immunologische effecten geïnduceerd door bestaande en nieuwe therapieën in kaart te brengen en om op basis van deze informatie nieuwe combinatie schema's met anti-PD-1 uit te testen.

2. Materiaal en methoden

Muis astrocytoma CT-2A neurosfeer (NS) cellen werden gegenereerd en intracraniaal geïnjecteerd in immunocompetente, vrouwelijke C57BL/6J muizen met een hoeveelheid van 5 x 10^3 levende cellen 0,5 mm anterior van de bregma, 2,5 mm lateraal van de middenlijn en 2,5 mm onder de dura mater gedurende 4 minuten. Muizen werden behandeld met RT, TMZ en/of anti-PD-1. Voor de RT behandeling werd telkens een enkele dosis van 4 Gy toegediend door middel van externe focale irradiatie op basis van magnetische resonantie beeldvorming (MRI) beelden, de TMZ behandeling werd 4x toegediend aan 50 mg/kg lichaamsgewicht via een orale gavage en anti-PD-1 behandeling bestond uit een intraperitoneale injectie (IP) van 100 µg, gedurende 3x. Muizen werden gerandomiseerd in verschillende behandelingsgroepen die zowel monotherapieën als combinatie-therapieën kregen.

Resultaten werden gegenereerd door middel van MRI, immuun monitoring van de tumor en de opvolging van de overleving. Voor survival analyses werden muizen opgevolgd wat betreft hun gewicht en neurologische symptomen. Tumor volumes werden manueel afgelijnd en berekend op basis van MRI scans (genomen voor en na de behandeling), en uiteindelijk gecorreleerd aan informatie verkregen uit flow cytometrie analyses afgenomen na voltooien van de behandeling. Voor flow cytometrie analyses, werden de muizen opgeofferd om vervolgens de immuun cellen uit de het hersenweefsel te kunnen isoleren op basis van een Percoll gradiënt. Cellen werden vervolgens gekleurd voor verschillende extracellulaire en intracellulaire markers voor T cellen (CD45, CD3, CD4, CD8 en FoxP3) en MF en MDSC cellen (CD11b, CD45, Ly6G, Ly6C, MHCII, F4/80 en CD206).

3. Resultaten

Zowel RT (p=0,004) als TMZ (p=0,004) in monotherapie, konden de overleving van muizen significant verlengen vergeleken met onbehandelde controle muizen, terwijl anti-PD-1 (p=0,2169) hier geen effect op had. Flow cytometrie analyse toonde aan dat, hoewel RT en TMZ beide een positief effect hadden op overleving in muizen, ze een tegenstrijdig immunologisch profiel

vertoonden. Een RT behandeling had een meer activerend effect op het immuunsysteem terwijl TMZ een sterk algemeen immuun suppressief effect teweegbracht. Opvallend genoeg had anti-PD-1 zeer weinig effecten op de kenmerkende immunobiologie van GBM.

In combinatie therapieën werden dezelfde parameters geanalyseerd. In een eerste groep werd het effect van RT, RT – TMZ en RT – TMZ – anti-PD-1 (behandelingen werden gegeven in de volgorde van vermelden) geanalyseerd. Het toevoegen van TMZ aan de RT behandeling verlengt de levensduur op een significante manier (p=0,0142) in proefdieren, overeenkomstig met het effect dat gezien wordt bij patiënten. De toevoeging van anti-PD-1 op het einde van deze behandeling, gaf een zekere trend naar een bijkomend gunstig effect (p=0,0842) en was in staat 20% van de behandelde muizen te genezen. Deze resultaten waren overeenkomstig met de effecten die gezien werden in de immuun micro-omgeving van de tumor. De RT – TMZ combinatie verlengde het leven van de muizen vergeleken met de RT behandelde muizen, echter dit wordt niet veroorzaakt door een gunstig effect op het immuun systeem maar door het directe cytotoxische effect van de behandeling. Dit kan ook teruggevonden worden in onze resultaten waar TMZ een onderdrukkend effect bleek te hebben op alle onderzochte immuun cel populaties. Vooral een neiging naar een reductie in $CD8^+/FoxP3^+$ ratio was opvallend (p=0,1302). Het toevoegen van anti-PD-1 was gedeeltelijk in staat dit negatieve effect om te keren richting een verhoging in $CD8^+/FoxP3^+$ ratio (p=0,0973). Een ander effect van anti-PD-1 is een schijnbare reductie in de pro-tumorigene M2 MF (p=0,1117) en mMDSCs, hoewel dit laatste niet ondersteund wordt door statistische analyse.

In het tweede deel van de combinatie experimenten, werden meerdere combinatie schema's met elkaar vergeleken. Er werden echter geen verschillen genoteerd tussen de verschillende combinatieschema's met anti-PD-1, zowel in overlevingsanalyses als in de immunologische benadering. Dit is een eerste indicatie dat het veranderen van het toedieningsmoment in het huidige behandelingsschema, geen aanvullend positief effect heeft op het totale therapeutische effect.

Muizen werden ook verdeeld in responders en non-responders op basis van het verschil in tumor volume dat gemeten werd door middel van MRI scans. Wanneer de behandelingen afzonderlijk toegediend werden, had de immunobiologie van responders een hoger totaal aantal

T cellen (p=0,0120) en $CD4^+$ T cellen (p=0,0477) en een reductie in immuun suppressieve $FoxP3^+$ T cellen (p=0,0006) en pro-tumorigene M2 MFen (p=0,0432) vergeleken met non-responders, wat het gunstige effect op het tumor volume duidelijk in verband brengt met een activerend effect op de immuuncellen aanwezig in de micro-omgeving van de tumor. Eenzelfde verdeling werd gemaakt voor muizen behandeld met de verschillende combinatie schema's. Hier werden echter compleet andere effecten waargenomen. In responders was er een duidelijke reductie in alle immuun cel populaties aanwezig in de tumor micro-omgeving, echter ook in de cellen die voor de positieve immuniteit kunnen zorgen. Vermoedelijk wordt dit veroorzaakt wordt door het toxische effect van TMZ op het immuunsysteem. De enige parameter die onaangeraakt bleef, was de $CD8^+/FoxP3^+$ ratio.

4. Discussie en conclusie

Ten eerste hebben wij aangetoond dat RT en TMZ, wanneer apart toegediend, beiden een positief effect hebben op de overleving van muizen terwijl dit effect niet gezien wordt in anti-PD-1 behandelde muizen. Uit immunologische analyse blijkt dat RT een sterk activerend effect heeft op het immuun systeem en TMZ een algemeen suppressief effect, wat laat blijken dat het positieve effect van TMZ op de overleving niet veroorzaakt wordt door een sterkere immuunactivatie maar eerder door het directe cytotoxische effect van het geneesmiddel. Opvallend is dat RT en TMZ tegenstrijdige effecten vertonen op het immuunsysteem waardoor TMZ in de behandeling van patiënten het immuunactiverende effect van RT in belangrijke mate neutraliseert. Anti-PD-1 vertoonde weinig of geen effecten op de immunobiologie van GBM in muizen, overeenkomstig met de afwezigheid van een effect op overleving.

Ook hebben wij aangetoond dat hetzelfde TMZ geïnduceerde immuun onderdrukkende effect aanwezig is in RT – TMZ behandelde muizen, de standaard behandeling in patiënten, en dat bijkomend voordeel op vlak van overleving dus voornamelijk afkomstig is van het cytotoxische effect van de chemobehandeling. Dit effect van TMZ zorgt voor een verlaging van de $CD8^+/FoxP3^+$ ratio wat een maat is voor de activatie van het immuun systeem. Het toevoegen van anti-PD-1 aan dit schema kon het effect op deze ratio gedeeltelijk omkeren, maar was niet genoeg om een

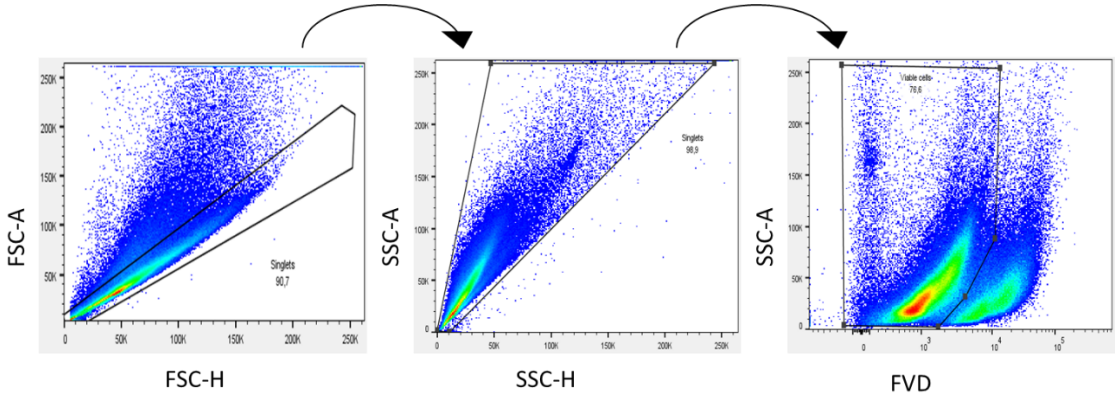
significant voordeel op te leveren op vlak van overleving hoewel er wel een trend waar te nemen was in die richting. Dit combinatieschema werd recentelijk ook getest in een fase III klinische studie (NCT02017717) waar Nivolumab (een anti-PD-1 monoclonaal antilichaam) de survival niet significant kon veranderen. Dit onderstreept de nood naar betere behandelingsschema's voor immuun checkpoint inhibitoren in GBM. Het aanpassen van het toedieningsmoment van anti-PD-1, blijkt momenteel geen invloed te hebben noch op overleving noch op verandering van de immunobiologie.

In onze experimenten met monotherapie, kon er duidelijk een shift waargenomen worden in immuun cel populaties van een meer immuunsuppressieve omgeving in non-responders en een meer immuunactiverende omgeving in responders, wat ook weergegeven wordt door het verschil in $CD8^+/FoxP3^+$ ratio in deze groepen. Dit onderstreept het belang van het immuun systeem in de respons op deze behandelingen in GBM. In tegenstelling tot deze resultaten, ging deze immuun stimulerende shift verloren in de responders afkomstig uit de combinatie experimenten. Wij denken dat dit verschil toe te schrijven is aan de schadelijke effecten die TMZ heeft op het immuunsysteem, waardoor het in zijn geheel uitgeschakeld wordt, en gedeeltelijk door de artificiële verdeling van responders en non-responders die in deze experimenten is toegepast.

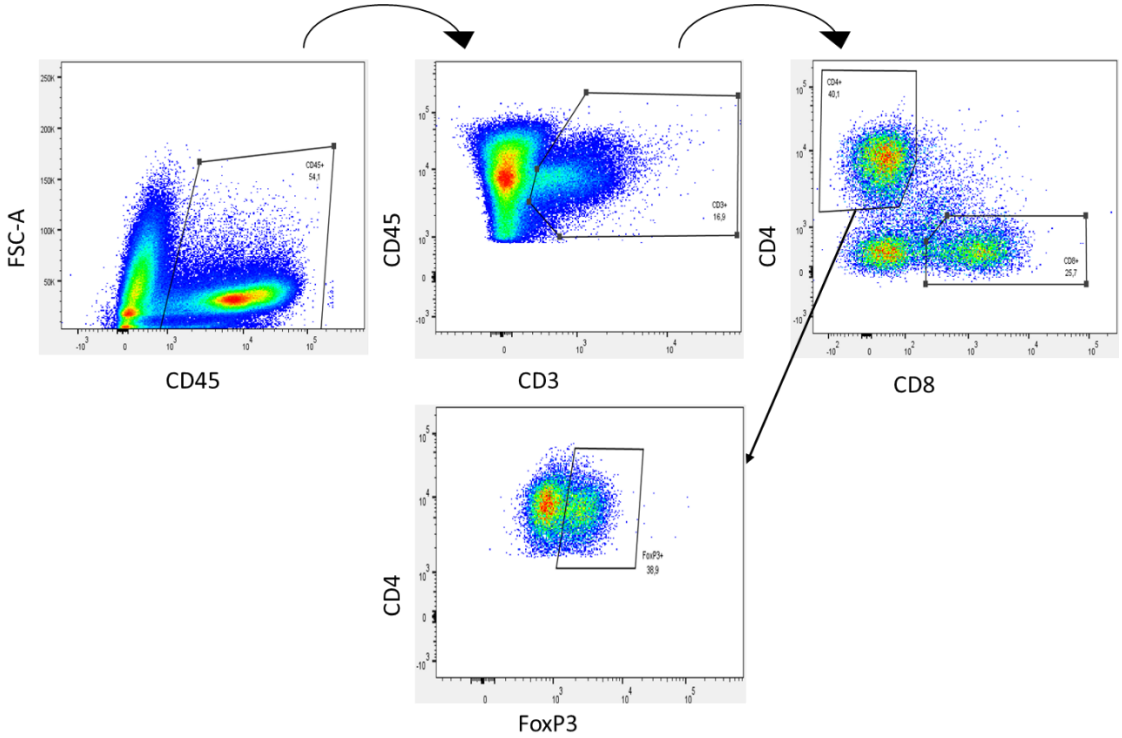
We kunnen concluderen dat de effecten van zowel conventionele als immunotherapeutische behandelingen op de immunobiologie van GBM van groot belang zijn in het opstellen van combinatieschema's en dat tot op heden anti-PD-1 de schadelijke effecten van TMZ op dit immuun systeem nog niet voldoende kan omkeren om te slagen in klinische studies. Er is dan ook dringend nood aan verbetering op vlak van combinatieschema's die op een meer synergistische wijze opgesteld moeten worden en alle individuele effecten van de verschillende behandelingen in acht nemen om het immuun systeem op een meer optimale wijze te activeren tegen de tumorcellen.

APPENDIX

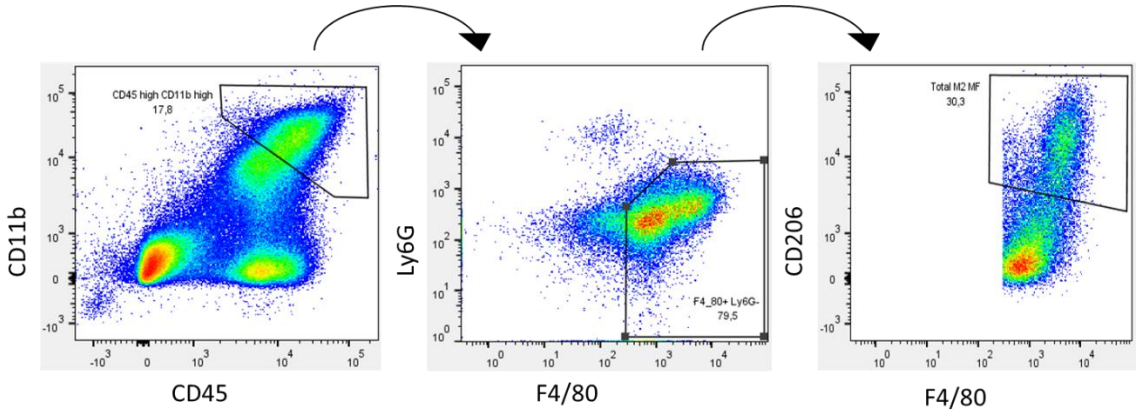
FlowJo gating strategy for single viable cells



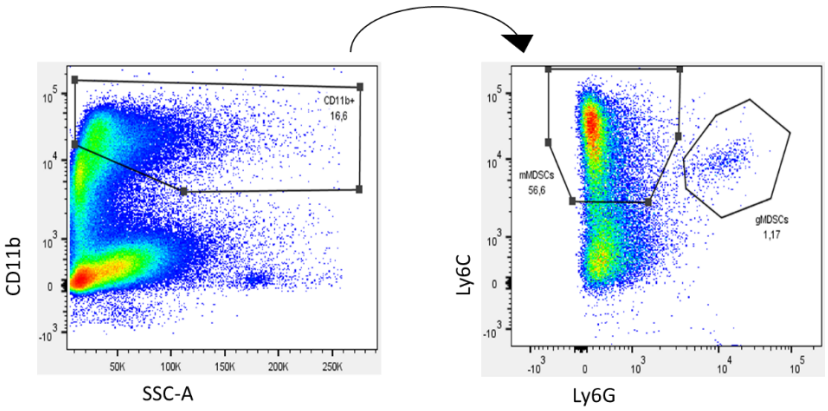
FlowJo gating strategy for T cell populations



FlowJo gating strategy for MF cell populations



FlowJo gating strategy for MDSC cell populations



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