UNIVERSITÀ DEGLI STUDI DI MILANO BICOCCA

SCUOLA DI DOTTORATO
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REFINING STRATEGIES FOR DENDRITIC CELL (DC) IMMUNOTHERAPY IN GLIOBLASTOMA PATIENTS

Dr. Cristina CORBETTA
Registration Number 722123

Tutor: Dr. Gaetano Finocchiaro
Co-Tutor: Dr. Serena Pellegatta

Coordinator: Prof. Andrea Biondi

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Alla mia famiglia

Table of Contents

Chapter 1 .... **7**

1. General Introduction **8**

1.1 Glioblastoma 8

1.2 The current state of GBM immunotherapy 11

1.2.1 The CNS: an immunologically specialized site 11

1.2.2 Immunotherapy approaches for GBM 14

1.3 Dendritic cell (DC) immunotherapy for GBM 17

1.4 Combined therapy for GBM: DC immunotherapy with adjuvant temozolomide and radiotherapy 24

1.4.1 Immunomodulatory effects of chemotherapy and radiotherapy. 24

1.4.2 Temozolomide 28

2. Scope of the thesis **32**

3. References **34**

Chapter 2 .... **49**

Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not $CD8^+$ T cell activation in the presence of adjuvant temozolomide **50**

Chapter 3 .... **81**

Expansion of effector and memory T cells is associated with increased survival in recurrent glioblastomas treated with dendritic cell immunotherapy **82**

Chapter 4 .... **119**

1. Summary **120**

2. Conclusions **123**

3. Future perspectives **129**

4. References **132**

5. Publications **135**

Chapter 1

1. General introduction

1.1 Glioblastoma

Brain tumors are rare cancers characterized by high morbidity and mortality due to their localization and high invasive growth[1]. The classification of these tumors is based on the *World Health Organization (WHO) 2000 Classification of Tumors of the Central Nervous System*, which assigns a grade (I to IV) according to the predicted clinical behavior[2].

Gliomas are the most common type of primary brain tumors (30%) and originate from neuroglial stem or precursor cells[2]. Glioblastoma (GBM) is the most common and lethal of all primary malignant central nervous system (CNS) tumors (47.1%), the most aggressive diffuse glioma (56.1%) of astrocytic lineage, and is considered a grade IV glioma based on the WHO classification[2]. It predominantly manifests in patient >50 years of age[1]: the incidence increases with age peaking at 75-84 years and it is 1.58 times higher in males compared to females[2]. Without treatment the median survival is of only 3 months[3]. Clinical presentation can vary depending on the size and localization of the tumor, but in general GBM patients may present symptoms of increased intracranial pressure, including headache, focal neurologic deficits, confusion, memory loss and saizures[4]. More than half of GBM patients die within one year from the diagnosis, and only 5% survive more than 5 years despite aggressive therapies[2]. GBM treatment remains dismally troubling even though great progresses in the management of the pathology. Currently, maximal safe total resection, radiotherapy and/or

chemotherapy with temozolomide (TMZ) remain the gold standard for newly diagnosed GBM, leading to an overall survival (OS) of about 15 months[5]. However, in spite of this multidisciplinary approach, about 70% of GBM patients will experience disease progression within one year of diagnosis[5]. In this scenario, GBM remains incurable due to its high potential for local invasion, neoangiogenesis and escape of the immune system that lead inevitably to a recurrence. Therefore, there is an urgent need of novel therapeutic strategies in order to delay this relapse.

Clinically, GBM is classified into primary and secondary, which constitute two distinct disease entities that develop through distinct genetic pathways[6] (**Figure 1**). The most frequent primary (*de novo*) GMB occurs in older patients (mean age = 62 years) without evidence of a less malignant precursor and progresses rapidly[6]. It is characterized by epidermal growth factor receptor (EGFR) amplification, $p16^{INK4a}$ deletion, PTEN mutation and completely loss of chromosome 10[7].

Secondary GBM is less common and develops in younger patients (mean age = 45 years) from initially low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III)[7]. TP53 mutations, loss of heterozygosity of 19q and retinoblastoma-associated protein RB1 loss are typically for secondary GBM[7]. Furthermore, isocitrate dehydrogenase 1 (IDH1) mutation is a genetic marker for secondary GBM, associated with improved survival and better outcome[6].

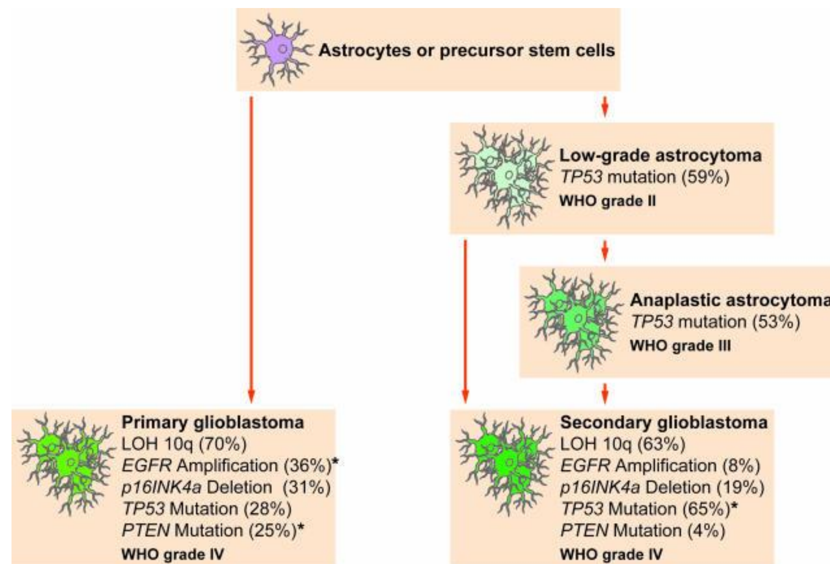

Figure 1 Primary and secondary GBM development. Mainly genetic alterations in primary and secondary GBM. *significantly different abnormalities in primary and secondary GBM[7].

The molecular heterogeneity of GBM has been unraveled leading to the identification of four different clusters: proneural, neural, classical and mesenchymal, each characterized by specific genetic alterations and expression profile[8,9]. This classification may help to focus available treatments and define new ones. Particularly, the proneural subtype is associated to a better prognosis and therefore longest OS related to the presence of the IDH1 mutation[10]. The mesenchymal subtype is the most aggressive characterized by the overexpression of many inflammatory-associated genes, which make it more immunogenic and therefore more responsive to immune-based therapies[11]. Moreover, it is established that the shift form proneural toward the mesenchymal phenotype is associated to tumor progression and therefore, to treatment resistance[10].

In 2016 the WHO classification of CNS tumors was revised, identifying three GBM groups: *1)* GBM IDH1-wild type (90% of

cases) including giant cell GBM, gliosarcoma and epithelioid GBM; *2)* GBM IDH1-mutant (10% of cases); and *3)* GBM, NOS (not otherwise specified), including tumors for which IDH evaluation cannot be performed[12].

1.2 The current state of GBM immunotherapy

1.2.1 The CNS: an immunologically specialized site

In the past, the CNS has long been considered an immunologically privileged system due to the presence of the blood-brain barrier (BBB), graft acceptance, lack of conventional draining lymphatics, low MHC expression and low T cell trafficking[13]. This ancient view has been dramatically changed over the last 20 years, and currently, the CNS is considered both immune competent and able to interact with peripheral immune system[14]. Several data indicate that dendritic cells (DCs) play a central role in starting the immune response in CNS[15]: they are recruited to the site of brain lesion from the periphery[16], where they mature and move back to the secondary lymphoid organs through cerebrospinal fluid and perivascular spaces and prime antigen-specific immune response[17]. Next to the DCs, perivascular macrophages and parenchymal microglia function as antigen presenting cells (APC) in the CNS in response to inflammatory or microbial stimuli[18]. The trafficking of T cell into the CNS is a highly regulated process, involving antigen specificity, upregulation of integrins and the generation of a chemotactic gradient, that all determine T cell BBB crossing, migration, antigen-specific reactivation and amplification of the immune response[17]. The long-

held concept of the absence of lymphatic vasculature in the CNS was subverted in 2015 by Louveau et al[19]. They identified functional lymphatic vessels lining the dural sinuses, a novel route of lymphatic egress from the brain. Thus, most APC exiting the brain travel to the deep cervical lymph nodes, where the can prime T and B lymphocytes[19].

The immune system is able to recognize and fight against cancer[20]. Consequently, the concept of immune escape, introduced by Hanahan and Weinberg, has long been investigated and is now recognized the new “Hallmarks of cancer”[21]. The process of “cancer immunoediting" is tightly related to this and develops in three main phases: *elimination*, *equilibrium*, and *escape* (**Figure 2**)[22]. It has been demonstrated that the absence of an active immune system leads to tumors grow faster in animal models[23]. Based on this concept, if the immune system can successfully fight cancer, we may try to re-educate lymphocytes to recognize tumors. In the last 10 years, a great effort has been made in order to extend immunotherapy to brain cancer and specifically to GBM.

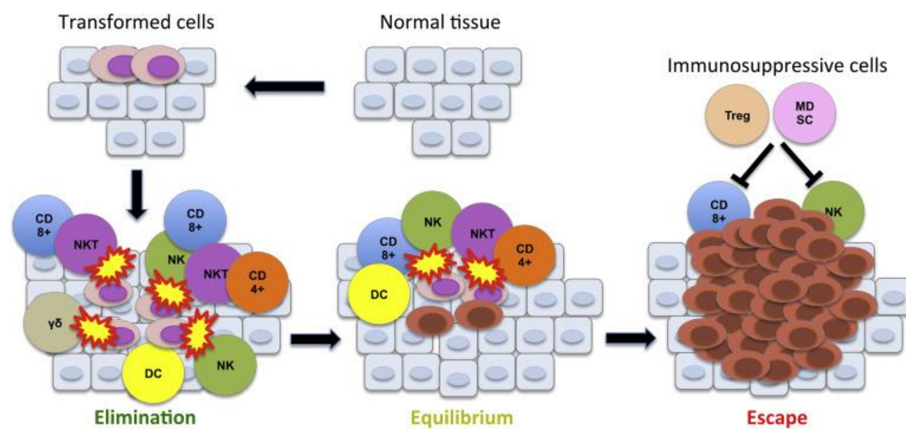

Figure 2 Immunosurveillance hypothesis describes the ability of the immune system to recognize and eliminate tumor cells. Immunoediting is a process triggered after the encounter between immune components and tumor cells. In the *elimination* phase transformed tumors cells may be recognized and eliminated by different types of immune cells. If elimination is unsuccessful, the immune system and cancer can reach an *equilibrium* phase, which involves the continuous elimination of tumor cells and the production of resistant tumor variants by immune selection pressure. This continuous sculpting can lead to *escape*, in which mutated cancer cells become able to inhibit the immune system and cancer can growth uncontrolled[24,25]**.**

Some evidence indicates that immune activation within GBM is suppressed by a strongly immunosuppressive tumor microenvironment[26], delineated by immunosuppressive cytokine production, T cell proliferation, and effector response inhibition, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) infiltration[27]. Particularly, GBM is characterized by the presence of a large number of *1)* immunosuppressive soluble factors, such as vascular endothelial growth factor (VEGF), interleukin 10 (IL10)[28], transforming growth factor beta (TGF-β)[29]; 2) immunosuppressive enzyme, such as indoleamine 2,3-dioxygenase (IDO)[30], cyclooxygenases (COX-2)[31], arginase and nitric oxide synthase-2 (NOS-2)[32]; *3)* immunoinhibitory molecules expressed on the surface of glioma cells, such as HLA-G and HLA-E[33,34],

programmed cell death ligand 1 (PD-L1)[35] and galectin-1 and 3[36,37]. The exact mechanism of GBM immune escape is unknown, although MDSCs and Tregs are key mediators of this process. MDSCs have been shown to favor cancer progression by dampening anti-tumor immune response, promoting angiogenesis, and creating pre-metastatic environment[38,39]. Tumor infiltration by myeloid cells is usually associated with poor clinical outcome[40,41]. Moreover, MDSCs can exert a negative effect on NK cells and T cells, impairing DCs activity[42]. On the other hand, the presence of a high number of intratumoral Tregs has been associated with high-risk relapse and poor overall survival in gliomas[43,44] and other cancers[24].

All these aspects are responsible for immunotherapy failure in GBM.

1.2.2 Immunotherapy approaches for GBM

Immunotherapy has provided a real breakthrough in cancer treatment, showing great clinical impact and, being a promising and attractive option also for GBM[45]. The field is rapidly expanding and currently, there are several approaches that have reached the phase III of clinical development, and numerous others at earlier stages[45].

Rindopepimut is a peptide vaccine that targets the EGFR variant III (EGFRvIII), a constitutively active mutant form of EGFR expressed exclusively in 25-30% of GBM patients[46]. The advantage of targeting EGFRvIII relates to its restricted expression on tumor cells, limiting the so-called “on-target, off-tumor toxicity”. However, this neoantigen is heterogeneously expressed, thus creating a potential for outgrowth of tumor cells that lack the antigen. Early studies evaluating the effect of rindopepimut vaccination in patients with GBM have demonstrated

increased median OS compared to controls[47]. These observations led to the initiation of an international phase III clinical trial, named ACTIV[48]. However, the trial was stopped earlier after an analysis revealing no significant difference in overall survival between the rindopepimut treated arm and the control one[48]. EGFRvIII was also proposed as a target for chimeric antigen receptor (CAR) T cell therapy. In 2017, data from the first-in-human clinical trial of autologous T cells redirected to EGFRvIII mutation by CAR indicated that the treatment was safe, but patients survival was not improved[49]. Nevertheless, deep evaluation of the tumor microenvironment demonstrated increased and robust expression of inhibitory molecules (e.g IDO1 and PDL1) and infiltration by Tregs after CAR T cells treatment[49], suggesting the need to overcome the immune escape mechanism carry out by GBM, in order to improve the effectiveness of this approach. This point out the need to target antigens with low intra- and inter-tumoral heterogeneity. Several studies at preclinical level, including our own, aimed to investigate the potential of new antigens as therapeutic target for GBM[50,51]. We have recently demonstrated that CAR T cells redirected to chondroitin sulfate proteoglycan 4 (CSPG4) efficiently controlled the growth of GBM cells *in vitro* and *in vivo* upon intracranial tumor injection. Overall, tumor necrosis factor alpha (TNF-α), released by microglia, up-regulated CSPG4 on tumor cells, thus reducing the risk of tumor escape[51].

A further promising immunotherapy approach for GBM is the use of immune checkpoint inhibitors, which have demonstrated encouraging clinical outcome in the treatment of patients with solid tumors

especially melanoma[52,53]. They are monoclonal antibodies directed against negative regulators of immune response, including cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 is only expressed on T cells and functions as an inhibitory receptor regulating the early stages of T cell activation; PD-1 is another inhibitory receptor expressed on activated T cells that regulate T cell activity in the effector phase within tissue and tumors[54]. Nivolumab, the anti-PD1 antibody, has been testing in the phase III CheckMate 143 clinical study in comparison with bevacizumab, but preliminary data revealed that the primary endpoint was not met and there was not a difference in terms of improved survival between the two cohorts[55]. Recent data have shown that response to checkpoint inhibitors is significantly more effective in the presence of hypermutations due to mismatch repair (MMR) deficiency and that a number of such mutations are neoantigens that may elicit immune responses[56,57].

Finally, another emerging promising therapeutic approach for GBM involves tumor-infiltrating lymphocytes (TIL). TIL-based therapy has demonstrated a good clinical outcome in metastatic melanoma: clinical data indicate that treatment with *ex vivo* - expanded TIL can result in 40% to 50% objective responses[58]. Recently, Zacharakis N et al. reported a complete tumor regression in a woman with advanced metastatic breast cancer treated with TIL[59], highlighting again the incredible potential of this kind of strategy. However, TIL – based therapy remains challenging for GBM due to the immune suppressive microenvironment that can influence the proliferation ability and the cytotoxic activity of infiltrating immune cells[60].

Dendritic cell (DC) immunotherapy has demonstrated promising results in GBM clinical trials. Since this approach assumes a relevant role in this thesis, it has been described in a dedicated paragraph (**1.3**).

1.3 Dendritic cell (DC) immunotherapy for GBM

Dendritic cells (DCs) were discovered 40 years ago by Ralph Steinman[61], who has been awarded the Nobel Prize for Medicine in 2011. DCs are a population of immune cells that provide a link between the innate and adaptive immune response and play also a crucial role in antitumor immunity[62]. Although they represent only a small fraction of leukocytes, they are the most powerful antigen-presenting cells (APC) with the unique ability to activate naïve T cells[63]. DCs take up antigens and present them to naïve T cells, that become potent effectors able to mediate a specific antitumor immune response[64,65]. However, this capacity of presenting antigen is limited by the presence of a strong immunosuppressive environment typical of glioma and other cancers[66]. Therefore, the presentation of cancer antigens becomes inefficient, by virtue of which scientists tried to re-present these antigens to DCs *in vitro*, and re-inject them back to patients.

The goal of DC immunotherapy is to activate tumor-specific effector cells able to reduce or eradicate the tumor mass and generate an immunological memory to control relapse[62]. Thus, the first step is to provide DCs with tumor-specific antigens. This may be achieved by culturing *ex vivo* DCs with the tumor-specific antigens, and then injecting them back into the patient, or by inducing DCs to take up the tumor-specific antigens *in vivo*[67].

Current clinical DC-based strategies exploit patients DCs to generate therapeutic vaccines. Particularly, DCs are harvested from patients, maturated *ex vivo*, loaded with tumor antigens and injected back into the patient, where they present tumor antigens to specific T cells (**Figure 3**)[68]. At the Fondazione IRCCS Istituto Neurologico Carlo Besta, where this doctoral thesis was performed, the Cell Factory has an optimized protocol to obtain DCs vaccines under good manufacturing practice (GMP) conditions[69]. Since circulating DCs represent only 0.1-1% of circulating peripheral blood lymphocytes (PBLs), the majority of DCs must be obtained *in vitro* from $CD14^+$ monocytes purified from leukapheresis[70]. Patient $CD14^+$ monocytes are obtained by immunomagnetic cell sorting using CliniMACS® device. Upon differentiation of $CD14^+$ with interleukin 4 (IL4) and granulocyte – macrophage colony – stimulating factor (GM-CSF), DCs are induced to maturation with TNF-α, IL1β, and IL6 and pulsed with the autologous whole tumor lysate, obtained in a closed system using the semi-automated dissociator GentleMACS®. The expression of tumor-associated antigens (TAAs) is very heterogeneous in GBM. The use of whole tumor cell lysate is advantageous because the identities of tumor antigens do not need to be known, the antigen repertoire is unique to the patient's tumor and the use of multiple tumor antigens reduces the risk of antigen-negative escape mutants. At the end of the process, mature and loaded DCs are frozen in batches and thawed at each vaccination[69,71].

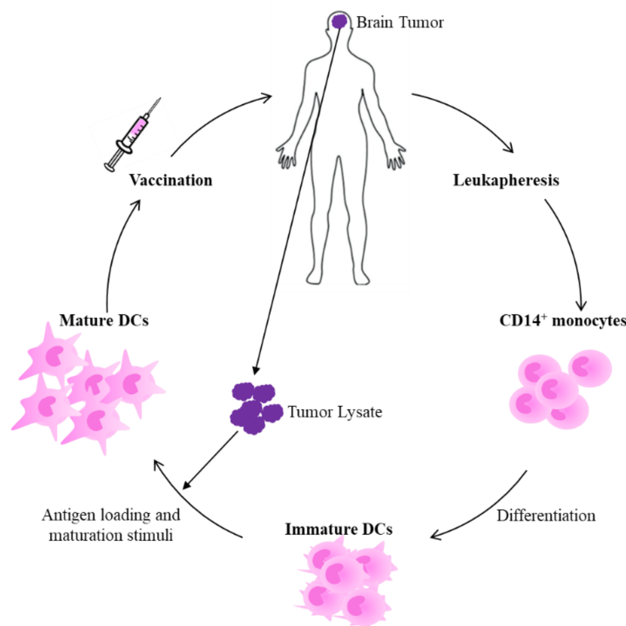

Figure 3 DC immunotherapy strategy. Patients undergo to apheresis and monocytes were isolated by immunomagnetic $CD14^+$ selection. Upon differentiation of $CD14^+$ with IL4 and GM-CSF, immature DCs are pulsed with the whole tumor lysate and induced to maturation with a pro-inflammatory cytokine cocktail. Finally, matured and loaded DCs are frozen and thawed at each vaccination.

Several groups, including our own, have worked many years on the development of DC-based therapeutic vaccine providing data on safety and efficacy in clinical trials[72–76]. In 1996 Hsu et al. published the first clinical study in which patients with B cell lymphoma were treated with antigen-pulsed DCs: all treated patients experienced measurable and specific antitumor immune response[77]. This approach was also used to treat prostate cancer patients in a phase III study, in which DCs were loaded with the prostatic acid phosphatase (PAP) peptide. The results of this study demonstrated improved patient survival and in 2010 the developed drug, Sipuleucel – T (Provenge), became the first FDA approved DCs vaccine for prostate cancer treatment[78].

Yu et al. demonstrated for the first time that autologous tumor lysate-pulsed DC vaccine was safe, feasible and able to generate an antigen-specific immune response in patients with malignant glioma[76]. Moreover, Yamanaka et al. showed that patient longer survival is correlated to tumor lysate-reactive $CD8^+$ T cells detection in the blood[79]. More recently, Liau et al. published the first data about the phase III DCVax®-L clinical study in patients with GBM, in which DCs were pulsed with the patient's whole tumor lysate, as a source of tumor antigens[80,81]. These data elucidated again the well-known safe and feasible profile of the DC-based vaccine, accordingly with an extended median overall survival of 23.1 months[81].
Two clinical studies, DENDR1 and DENDR2 (DENDR1-EUDRACT No 2008-005035-15; DENDR2-EUDRACT No 2008-005038-62), including respectively the treatment of first diagnosis and recurrent GBM patients with DCs loaded with autologous tumor lysate have been activated at the Fondazione IRCCS Istituto Neurologico Carlo Besta. Currently, the DENDR2 study is closed due to lack of significant anti-tumor immune response and survival advantage; by contrast, the DENDR1 study is still active. In both studies, DC immunotherapy was combined with chemotherapy and TMZ is administered as a potential adjuvant.
The first results on a group of recurrent GBM demonstrated a correlation between increased PFS and OS and higher frequency and activation of NK cells[82]. Investigating serum levels of immunosuppressive factors (e.g. TGF-β and VEGF) we founded an inverse correlation with patient survival, stressing the role the immunosuppressive microenvironment in limiting the efficacy of DC

immunotherapy. On the other hand, we identified a positive correlation between PFS and IL12, a cytokine involved in IFNγ production by NK cells[82]. In the DENDR1 study we have observed that immunotherapy with DCs was able to induce a significant anti-tumor immune response[83,84]. However, only a subgroup of patients benefited from the chemo-immunotherapy combination and the gain in survival was associated with a specific and long- lasting NK, and not $CD8^+$ T, cell response. Specifically, TMZ impaired the anti-tumor T cells response having a negative impact on the generation of $CD8^+$ T cell memory status[84].

Although DC immunotherapy has proven to be safe and efficient, there is not yet convincing evidence of efficacy, which seems limited by several factors. Only 15.6% of patients with malignant glioma have an objective response to DC-based therapy[42]. Among the limiting aspects, we found:

- methods of preparation and loading of antigens to DCs;
- culture methods and maturation cocktails used for generating DCs;
- number of DCSs administered;
- route of DCs administration;
- capacity of DCs to migrate to lymph nodes (LNs);
- DCs ability to attract, interact with and induce the right kind of immune cells;
- ability of induced effector cells to home to tumors and eliminate them;
- the negative impact of the tumor microenvironment on DCs function and phenotype[85,86].

Overall, the ability of DCs to migrate to LNs is a very critical aspect affecting the success of the therapy, as it allows DCs to interact with and activate the adaptive immune cells[87]. Indeed, the number and the proportion of injected DCs that migrate to draining LNs directly affect the T cell priming. It is well established that the efficiency of DCs migration from the injection site to LNs is very low and less than 4-5% of injected DCs reach the LNs[88]. This constraint has prompted the evaluation of several approaches to enhance the migration of DCs, therefore improving the efficacy of DC immunotherapy. Preclinical data revealed that pre-conditioning the vaccine site with inflammatory cytokines or mature DCs significantly increased the migration of subsequent injected DCs to LNs, enhancing a $CD4^+$ T cell response[89]. In 2015 Mitchell et al. explored a new approach to pre-condition the vaccine site with the recall antigen tetanus/diphtheria toxoid (Td)[90]. In a small phase I study, they treated GBM patients with DCs loaded with RNA encoding *Cytomegalovirus* proteins (CMV pp65 RNA-pulsed DCs) and demonstrated that the Td pre-conditioning enhance LNs homing of DCs and consequently, the efficacy of tumor-antigen-specific DCs[90]. Moreover, the unilateral administration of Td produced a recall response able to increase the bilateral migration of DCs in both patients and mice. This leaded to an increase in PFS and OS in treated patients. Furthermore, investigations in preclinical mouse model revealed that the chemokine (C-C motif) ligand 3 (CCL3), produced by $CD4^+$ T cells, was responsible for this response[90] (**Figure 4**).

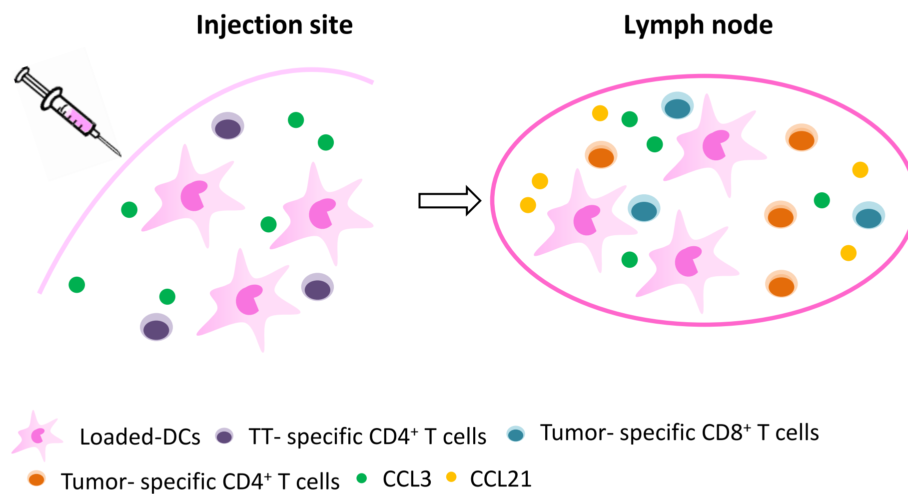

Figure 4 Vaccine site pre-conditioning to facilitate LNs homing of DCs. Td injected at the vaccine site enhances DCs migration by inducing inflammatory immune responses mediated by Td-recognizing CD4$^+$ T cells and generation of the protein CCL3. This protein up-regulates the production of the protein CCL21, which promotes DCs and T-cell migration into lymph nodes. CCL3 may also recruit CD8$^+$ T cells to sites where DCs and CD4$^+$ T cells interact (Adapted from[86]).

At preclinical level, several adjuvants have been employed to improve the immunogenicity of administered tumor-specific DCs by inducing a local inflammation[91–93].

Tetanus toxoid (TT) is a clinically approved vaccine with a very safe profile; it may produce a recall immune response in patients and create an inflammatory stimulus at the vaccine site[94]. Furthermore, TT is able to induce a local delayed-type hypersensitivity (DTH) response that activates injected DCs and promotes their T cell stimulating functions[95]. A phase I clinical study of DC immunotherapy combined with TT for colorectal cancer treatment revealed an increased tumor-specific immune response and a clinical benefit[95]. Moreover, previous data suggested that a CD4$^+$ T population of cells, defined bystander, is

activated after the recall tetanus vaccination and displayed typical features of central memory T cells[96].

1.4 Combined therapy for GBM: DC immunotherapy with adjuvant temozolomide and radiotherapy

1.4.1 Immunomodulatory effects of chemotherapy and radiotherapy

Even conventional anticancer agents used in the clinic were selected for their immunosuppressive properties and their ability to rapidly killing proliferating cells, they demonstrated a limited capacity to eradicate tumors since patients undergo frequently to relapse. Radiotherapy (RT) has long been used for cancer treatment too, inducing cell death trough irreparable DNA damage[97]. Nevertheless, some tumors, including GBM, are still resistant to ionizing radiation effect[98]. Moreover, it has been previously debated how immunotherapy, as single therapeutic modality, has revealed not so effective and rarely curative. In this scenario, several studies are currently investigating the correct combination and schedule of radio – chemo - immunotherapy able to stimulate a systemic and local immune response that may keep residual tumor cells in check. This combination of immunotherapeutic strategies with conventional chemotherapy and RT confers several advantages[99,100].

In the past, chemotherapy and RT were considered immunosuppressive approaches for cancer treatment, but now a growing body of evidence supports their positive immunological effects[101]. They are able to mediate a strong antitumor response by

inducing the so-called "immunogenic death" of tumor cells (ICD) modulating tumor microenvironment or depleting immunosuppressive immune cells[102–104]. It has been demonstrated in preclinical experiments that mice exposed to several chemotherapeutics agents experienced ICD[105]. ICD is often characterized by both morphological and biochemical features of apoptosis[106] and is able to convert succumbing tumor cells to a therapeutic vaccine stimulating an antitumor immune response[107,108]. At a pre-apoptotic stage, the chaperone calreticulin (CRT) translocates from the endoplasmic reticulum to the cell surface in response to death-signal[109,110] and acts as "eat-me" signal for macrophages[111], thus improving tumor antigen uptake by DCs[109]. Interfering with CRT translocation dramatically impacts on the immunogenicity of cell death, as highlighted by several preclinical studies[109,112], thus stressing the importance of this checkpoint for ICD. Later, the non-histone chromatin protein high-mobility group protein B1 (HMGB1) is secreted, thus stimulating antigen processing and presentation to T cells[113]. HMGB1 is a potent pro-inflammatory stimulus[114] released by dying cells that can bind to Toll-like receptor 4 (TLR4)[113]. Preclinical data showed that depletion of TLR4 and/or HMGB1 abolished tumor antigen presentation by DCs[113], indicating HMGB1 as another critical determinant of ICD. Finally, ATP is released, leading to the inflammasome activation and pro-inflammatory cytokines production[115]. ATP secreted from dying cells act as a "find-me signal" for DCs precursors, through P2Y2 receptor binding[116]. ATP effects are mediated partially by P2RX7 receptors binding, which activate the NLRP3 inflammasome and IL1β and IL18 production[115]. Interestingly, IL1β is determinant for the

recruitment and the maturation of tumor antigen-specific CD8^{+} T cells[117]. All these aspects are summarized in **figure 5**.

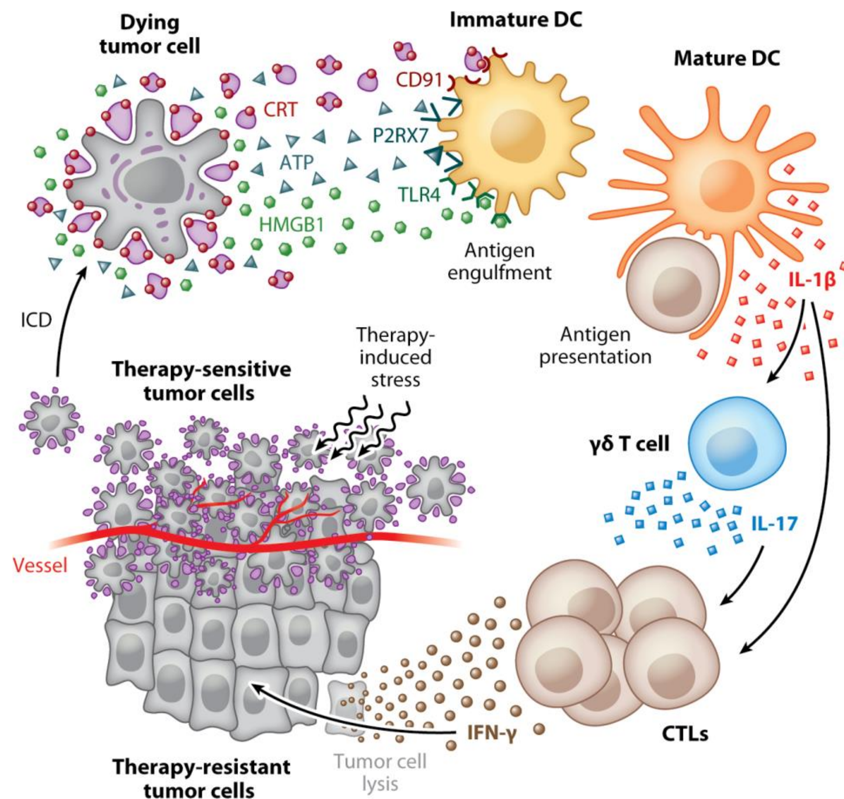

Figure 5 Immunogenic cell death (ICD) induced by chemotherapy. Following ICD cancer cells release soluble mediators that stimulate DCs to present tumor antigens to T cells. This process results in an antitumor immune response mediated by IFNγ, which eventually lead to tumor eradication (Adapted from[118]).

Chemotherapy and RT can act directly on tumor microenvironment, creating a more favorable milieu for the activation of an antitumor immunity[119]. It has been demonstrated that the immune infiltrate has a pivotal role for patients prognosis[120] and changes in its composition may be beneficial for cancer eradication[121]. A number of data indicates that several anticancer drugs increase the number and the antitumor activity of CD8^{+} T cells and NK cells[122,123]. Metronomic

chemotherapy is able to recruit NK cells to the tumor site mediating an immune response against tumors[124,125]. Moreover, it has been demonstrated that several tumor cell lines overexpressed NKG2D ligands, important for NK cells activation, following radiation[126]. CXCL16 production and adhesion molecules (e.g. L-selectin and ICAM1) up-regulation by RT mediated the recruitment of $CD8^+$ T cell into tumor[127,128].

Myeloid-derived suppressor cells (MDSCs) have a pivotal role in chemo – and radio-resistance. These cells are rapidly recruited to tumor site trough CSF1/CSF1R signal following radiation[129] and CXCL1/2 secretion by chemotherapy-exposed cancer cells[130]. On the other side, preclinical studies in mice showed that high dose radiation in combination with anti-PDL-1 reduced the accumulation of MDSCs and increased $CD8^+$ T cell infiltration in tumors[131,132]. It has also been demonstrated that several anticancer agents reduce MDSCs in cancer patients[133,134].

Tumor-associated macrophages (TAMs) are classified into M1 (tumor-cell-killing) and M2 (tumor promoting)[41]. Preclinical data indicated that chemotherapy promoted the shift from M2 to M1 macrophages, subverting their pro-tumorigenic activity[135]. Furthermore, RT impacts on the expression of chemokines released from TAMs, thus altering the control of T cell infiltration[136]. Several efforts have been made also in GBM, in order to damp macrophages mediated immunosuppression, thus favoring an antitumor $CD8^+$ T cell respose[137].

Several clinical data, obtained also in patients with GBM[138], have demonstrated a strong presence of Tregs in the tumor

microenvironment in response to radiation, thus highlighting their intrinsic radioresistance[139,140]. However, there are contradictory results indicated that low-dose irradiated mice decreased the number of Tregs, which was correlated to anti-tumor immune response activation[141]. Also chemotherapy has been shown to decrease Tregs number in tumor microenvironment[142].

Taken together, these findings indicate that the knowledge of how chemotherapy and radiotherapy influence the immunosuppressive nature of the tumor can be useful to exploit the full potential of immunotherapy for cancer treatment. Chemotherapy, radiotherapy and immunotherapy may cooperate to counteract immunosuppressive microenvironment and evoke an efficient and specific antitumor immune response.

1.4.2 Temozolomide

The oral alkylating agent temozolomide (TMZ) is a lipophilic prodrug, which is metabolized to its active form MTIC (5-(3-dimethyl-1-triazenyl) imidazole-4-carboxamide) at physiologic pH. After oral administration, TMZ is rapidly absorbed and the bioavailability is approximately 100%. During DNA replication, MTIC adds a methyl group to the N^7 and O^6 position of guanine and N^3 position of adenine. However, TMZ cytotoxicity is mainly mediated through the methylation of O^6 guanine that leads to thymine incorporation rather than a cytosine. The result is a base pair mismatch that causes apoptosis[143].

In 2002 Stupp published the first results of a pilot phase II study, proposing a concomitant RT plus TMZ (75mg/m^2/day) treatment

followed by adjuvant TMZ (150-200 mg/m^2 5 days on/23days off). Data indicated a median survival of 16 months[144]. This study was further followed by a larger phase III study, which demonstrated a significantly improved median survival (12.1 versus 14.6 months) in GBM patients treated with radiotherapy plus concomitant TMZ[5]. Overall, these data paved the way to the use of radiation with concomitant TMZ as a gold standard approach for GBM management. Several data indicated that patients with methylation of O^6-methylguanine-DNA-methyltransferase (MGMT) gene in tumors had greater benefit from TMZ treatment[145]. MGMT gene encodes for O^6-alkylguanine-DNA-alkyltransferase (AGT), a DNA repair enzyme that restores the damage induced by TMZ. When MGMT is methylated, its expression is reduced, thus the DNA damage is not repaired[143]. In virtue of this evidence, MGMT methylation status can predict response to TMZ and indeed is correlated with a better survival after TMZ administration[145,146].

Even though TMZ is safe and well tolerated for its low toxicity, its major side effect is lymphopenia[147]. Particularly TMZ has a great impact on bone marrow cells, which are characterized by lower MGMT activity than tumors. Several studies have shown that GBM patients treated with TMZ and radiation primarily experienced $CD4^+$ T and B, but also $CD8^+$ $CD56^+$ effector T, cells decrease[148]. Although counterintuitive, lymphodepletion induces a homeostatic lymphocyte proliferation that enhances antitumor immunity associated with immunotherapy[149]. Sampson et al. demonstrated that immunotherapeutic targeting of EGFRvIII significantly increased

antigen-specific immune response despite a great lymphopenia induced by dose-dense TMZ in phase II clinical study[149].

Moreover, TMZ can synergize with immunotherapy as demonstrated by Sanchez-Perez et al[150]. They showed that myeloablative doses of TMZ combined with immunotherapy led to a considerable expansion of antigen-specific $CD8^+$ T cells, supported by IL2 serum levels elevation, in tumor-bearing mice[150]. In addition, in lymphopenic condition, TMZ increases the abundance of cytokines and proliferation factors for remaining lymphocytes. However, IL7 and IL15, which are necessary for $CD8^+$ T cell proliferation and effector function, do not augment after TMZ[151]. Thus the remaining lymphocytes do not need to compete for the cytokines[151], and there is a reduction in T cell activation threshold and a proliferation induction. This homeostatic proliferation causes an increase in lymphocytes effector function that finally potentiates antitumor immune response[152].

TMZ improves also the cross-priming of tumor antigen-specific T cells: drug exposure releases tumor antigens that are captured by DCs and presented to T cells through MHC class I. In a GL261 mouse model, the combination of TMZ with DC immunotherapy enhanced the antitumor immune response through increased cross-priming mediated by calreticulin exposure[153].

It has been mentioned before that MDSCs and Tregs, found in GBM microenvironment, exerted a potent immunosuppressive activity that influenced the therapeutic response of patients. It has been documented that MDSCs treated with TMZ activate p53, p21 and γ-H2AX inducing an intrinsic mitochondrial pathway of apoptosis[154].

However, the effect of TMZ on MDSCs has not been confirmed in human patients[152]. Several clinical studies have demonstrated that TMZ alone is not enough to deplete Tregs. Mitchell et al. showed increased Tregs proportions after TMZ treatment in human and mice[155]. However, IL2 receptor (CD25) blockade reversed this scenario, reducing Tregs and enhancing the immune response to vaccine treatment[155]. Therefore, a great number of data indicate that Tregs depletion improve immunotherapy efficacy, but this cannot be achieved by TMZ alone[152].

These findings demonstrate that even though TMZ induces lymphopenia, it efficaciously exerts antitumor effect together with a significant impact on the host immune system. Moreover, vaccine strategies may favor antigen-specific T cell proliferation and function following lymphopenia. The timing and dose are critical for TMZ impact on tumor microenvironment, thus combinatorial strategies involving TMZ and immunotherapy will require thoughtful consideration to ensure optimal outcomes[152].

2. Scope of the thesis

Glioblastoma (GBM) is the most lethal adult cancer. Conventional standard of care treatment, including surgery, radio – and chemotherapy with temozolomide (TMZ), does not results in improvements in survival of patients.[5] Thus, the treatment of this pathology remains dismally troubling.
Cancer immunotherapy has emerged as the breakthrough against cancer, revolutionizing the clinical management of several tumors. Several immunotherapeutic approaches have been developed also for GBM with encouraging results. However, only a fraction of patients benefits from this kind of treatment mainly due to the highly immunosuppressive microenvironment and tumor antigen heterogeneity[45].

Two clinical studies, including respectively the treatment of first diagnosis (DENDR1) and recurrent GBM (DENDR2) patients with DCs, loaded with autologous tumor lysate, have been activated at Fondazione IRCCS Istituto Neurologico Carlo Besta. Given that chemotherapy has been proposed as an adjuvant able to influence the immune response, by inducing the immunogenic death of tumor cells or by modulating key cells for immune suppression or activation[118], in both studies the immunotherapy was combined with TMZ as an adjuvant.

In <u>**Chapter 2**</u> **"Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not $CD8^+$ T cell activation in the presence of adjuvant temozolomide"**, the first goal was to analyze the impact of the

combination of TMZ and DC immunotherapy on patients' immune response. We focused on peripheral blood lymphocytes and characterized the immune response with particular attention on NK cell population. Indeed our previous data indicated that increased survival in patients with recurrent GBM was primarily associated with NK cell response[82].

Data obtained from the DENDR1 study indicated that TMZ limited $CD8^+$ T cell activation and memory generation, thus the definition of the best-combining approach is still challenging. Recently, Mitchell and colleagues pointed out the role of vaccine site pre-conditioning in improving DC immunotherapy efficacy[90]. On the basis of this evidence, the pilot study variant (V) – DENDR2 aimed to evaluate the impact of tetanus toxoid (TT) pre-conditioning in absence of TMZ on patients anti-tumor immune response.

In **Chapter 3** **"Expansion of effector and memory T cells is associated with increased survival in recurrent glioblastomas treated with dendritic cell immunotherapy"**, we investigate whether the new strategy was able to activate specific immune effector cells involved in a long-term response and in a clinical benefit. Particularly, we focused on $CD8^+$ and $CD4^+$ T cell responses, that were absent in DENDR1, in terms of specific activation and memory generation.

Taken together the results will allow improving our knowledge about the effect of combinatorial approaches on antitumor immune response, thus encouraging standard schedule re-evaluation.

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Chapter 2

Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not $CD8^+$ T cell activation in the presence of adjuvant temozolomide

Serena Pellegatta*, Marica Eoli*, Valeria Cuccarini, Elena Anghileri, Bianca Pollo, Sara Pessina, Simona Frigerio, Maura Servida, Lucia Cuppini, Carlo Antozzi, Stefania Cuzzubbo, **Cristina Corbetta**, Rosina Paterra, Francesco Acerbi, Paolo Ferroli, Francesco DiMeco, Laura Fariselli, Eugenio A. Parati, Maria Grazia Bruzzone & Gaetano Finocchiaro

* These authors contributed equally to this work.

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Abstract

In a two-stage phase II study, 24 patients with first diagnosis of glioblastoma (GBM) were treated with dendritic cell (DC) immunotherapy associated to standard radiochemotherapy with temozolomide (TMZ) followed by adjuvant TMZ.

Three intradermal injections of mature DC loaded with autologous GBM lysate were administered before adjuvant TMZ, while 4 injections were performed during adjuvant TMZ. According to a two-stage Simon design, to proceed to the second stage progression-free survival (PFS) 12 months after surgery was expected in at least 8 cases enrolled in the first stage. Evidence of immune response and interaction with chemotherapy were investigated. After a median follow up of 17.4 months, 9 patients reached PFS12. In these patients (responders, 37.5%), DC vaccination induced a significant, persistent activation of NK cells, whose increased response was significantly associated with prolonged survival. $CD8^+$ T cells underwent rapid expansion and priming but, after the first administration of adjuvant TMZ, failed to generate a memory status. Resistance to TMZ was associated with robust expression of the multidrug resistance protein ABCC3 in NK but not $CD8^+$ T cells. The negative effect of TMZ on the formation of T cell-associated antitumor memory deserves consideration in future clinical trials including immunotherapy.

Introduction

Immunotherapy with dendritic cells (DC) has not yet fulfilled the promise for cancer treatment. Even if DC vaccinations have scarce toxicity and serious adverse events were not reported, clinical relevance has been hampered by the limited translation of immune activation into clinical response[1,2].

The observation that chemotherapeutic agents like anthracyclines may exert immunostimulatory effects has generated new expectations for DC immunotherapy of cancer[3–5].

Clinical data on the combination of temozolomide (TMZ), with immunotherapy are limited. In vitro results suggest that TMZ, the standard chemotherapy in glioblastoma (GBM), reduces expression of the T regulatory (Treg) cell attractant CCL2 in GBM cells[6,7]. In one patient TMZ-induced lymphopenia was synergistic with a peptide vaccine, possibly because of Treg inhibition[8]. In a rat model of GBM, TMZ depletes Treg cells using a “metronomic” schedule, and metronomic cyclophosphamide favors anti-glioma $CD8^+$ T cell responses[9,10]. Recent data in the mouse model GL261, however, showed that alkylating chemotherapy with a schedule resembling standard rather than metronomic treatment, impairs adaptive immune responses[11].

Overall, clinical data on the combination of TMZ and DC immunotherapy are lacking. Here we report the results of the first stage of DENDR1 (EUDRACT N° 2008-005035-15), a phase II, uncontrolled, open label, nonrandomized study in patients with first diagnosis of GBM in which DC immunotherapy is associated with standard radiotherapy and chemotherapy with TMZ.

Results

Patient treatment and survival

The schedule of the treatment and clinical features of the 24 patients are summarized in **Fig. 1A** and **Table 1**. Sixteen patients completed all scheduled vaccinations; 7 discontinued TMZ and immunotherapy after at least 4 vaccinations because of disease progression; one patient received 6 vaccinations, experienced severe pulmonary embolism and withdrew his consent to immunotherapy.

Twenty-two patients underwent recurrence, one died because of heart failure before progression (Pt 3) and one was still progression-free at the time of the analysis (Pt 23). Six patients with recurrence did not receive any treatment because of severe clinical worsening (Pts 5, 11, 18 and 19), while two were lost to follow-up (Pts 1 and 13). Eight of the other 16 patients had second surgery. Other treatments included bevacizumab (eight cases), chemotherapy (six cases), cyber-knife plus chemotherapy (one case) and radiotherapy (one case) (**Table 1**). Median follow up was for 17.4 months, median PFS was 10.5 months (95% CI 9.15-15.44), PFS6 was 79% and PFS12 41% (**Fig. 1 B**). Nine patients were free from progression at 12 months, thus satisfying the criteria we had set for passage from stage I to stage II of the Simon[12] design employed in this study. Here, for brevity and clarity we defined them as responders which does not imply that all nine had evidence of response to immunotherapy.

Two of 24 patients were alive at the time of analysis: median OS was 20.1 months (95% CI: 12.5-25), OS at 12 and 24 months were 75% and 37%, respectively (**Fig. 1C**).

Disease progression was suspected for 5 patients (Pts 10, 11, 15, 17, 19) based on MRI performed one week after the end of concomitant radiochemotherapy. These five patients were treated and included in survival analysis: as the following MRI confirmed progression, the time of disease progression was back-dated to previous MRI. Hypermethylation of the MGMT promoter, present in 25% of patients (4 responders and 2 non-responders), was the only clinical feature associated with longer PFS and OS (*P*=0.02 and *P*=0.03 respectively). The median PFS was 17.2 months (CI 95% 7.9-28.3) in the presence of MGMT methylation and 10.2 months in the other cases (CI 95% 7.9-14.7) (**Fig. 1D**). The median OS was 32.8 months (95% CI 20.2-33.9) in patients with MGMT methylation and 17.8 months in the others (CI 95% 14.4-22.6) (**Fig. 1E**). Exemplificative MRI of one responder and one non-responder (patients number 12 and 17, respectively) are shown in **Fig. 2**.

Vaccine safety and adverse events

Three intradermal injections of mature DC loaded with autologous whole tumor lysate were administered before adjuvant TMZ, 4 further injections were performed during adjuvant TMZ (**Fig. 1A**). The treatment was well tolerated. One patient stopped treatment before disease progression due to pulmonary embolism. One patient died before progression because of deep venous thrombosis and pulmonary embolism. One case of grade 5 disseminated intravascular coagulation (DIC) was reported.

Five cases of partial seizures, 8 convulsions and 1 myositis were also recorded. Non-serious skin reactions included itching, erythema, urticaria and temporary inflammation at the injection site. A list of

adverse events occurred during immunotherapy with relative grades is provided in **Table S1**.

Radio-chemotherapy and adjuvant chemotherapy affect CD8$^+$ T, CD4$^+$ T and NK cell counts

TMZ-induced lymphodepletion has been associated with expansion of a specific anti-tumor immune response[13]. We measured the absolute lymphocyte counts (ALCs) at leukapheresis (the basal time point), during and at the end of the treatment. Basal ALCs were >1,000 cells per μL peripheral blood in 22/24 patients at leukapheresis, and dropped significantly after RT-TMZ (from 1710.9 ± 753.9 to 726.0 ± 276.3, $P<0.0001$) (**Fig. 3A**). RT-TMZ induced significant lymphopenia (<1000 lymphocytes/microl) in 19/24 patients (79%). In 6/19 patients, the ALCs were < 500 after RT-TMZ.

RT/TMZ decreased CD8$^+$ T cell (499.8 ± 318.9 before RT/TMZ to 279.9 ± 165.4, $P = 0.004$), CD4$^+$ T cell (from 708.0 ± 371.8 to 312.6 ± 107.7, $P < 0.0005$) and NK cell counts (from 88.0 ± 72.9 to 45.7 ± 37.9, $P = 0.02$) (**Fig. 3B-D**).

We also investigated the absolute count of CD8$^+$ T, CD4$^+$ T and NK cell subsets in the peripheral blood of all patients before, during and after immunotherapy. The CD8$^+$ T cell subset of responders only increased early after the second and third vaccination, but decreased in combination with adjuvant TMZ (**Fig. 3E**). CD4$^+$ T cell counts did not increase during vaccinations (**Fig. 3F**). On the contrary, the NK cell subset of responders increased significantly after the third vaccination and remained constant over time (**Fig. 3G**).

DC vaccines induce early NK cell activation followed by CD4$^+$ T cells in a later phase

To investigate further the immune responses induced by DC vaccinations, we correlated counts and frequency of the 3 lymphocyte subsets with PFS and OS. ROC curve analysis identified 2.2 as the vaccination/baseline threshold (as defined in Materials and Methods, Statistical analyses) to differentiate patients with high or low CD8$^+$ T, CD4$^+$ T and NK cells (sensitivity 88.9%, specificity 80%). V/B ratios higher than 2.2 for NK but not for CD8$^+$ and CD4$^+$ T cell counts were associated with prolonged PFS and OS (median PFS 16.1 months vs. 9.3 months, P = 0.002; median OS 32.8 months vs. 17.8 months, P = 0.003) (**Fig. 4A, B** and **Fig. S1A, B**). This was confirmed by correlating survival data with NK, CD8$^+$ and CD4$^+$ cell frequencies (**Fig. S1C-E**). When the Bonferroni method was used for multiple testing, the differences remained significant (P=0.016 for PFS and P=0.024 for OS).

In order to investigate their independent prognostic role, a multivariate analysis and a Cox proportional hazard regression model analysis were performed on variables showing statistically significant differences at univariate analysis (i.e. MGMT methylation and V/B ratio for NK cell counts). Increased V/B ratio for NK cell counts remained the only significant predictor of PFS (P = 0.02, exp(b) 0.24, 95% CI 0.071 - 0.85) and OS (P = 0.03, exp(b)0.27, 95% CI 0.081-0.89).

To assess the specific anti-tumor immune responses induced by DC vaccinations we measured IFN-γ (**Fig. 4C, D**) and granzyme-b (GZMB) (**Fig. 4E, F**) expression by intracellular staining and flow

cytometry on NK cells, $CD8^+$ T in 11 patients (5 responders and 6 non-responders) with all blood specimens available (**Fig. 4C-D, Fig. S2A, B**). After two DC vaccinations a significant expansion of NK cells expressing IFN-γ in Peripheral Blood Lymphocytes (PBLs) was detected in responders (P = 0.01 vs. leukapheresis; P = 0.02 vs. first vaccine). After the third vaccine NK cells continued to express higher levels of IFN-γ in response to and at the end of treatment (P = 0.004, responders vs. non-responders). Notably, responders showed higher IFN-γ expression by NK cells and $CD8^+$ T cells already before RT-TMZ (2.9±1.2 vs. 0.6±0.7; P=0.01; 13.1±1.6 vs. 5.2±2.5; P=0.008, respectively, responders vs. non-responders). IFN-γ expressing $CD8^+$ T cells in responders showed an early and significant expansion after the second vaccine, but a rapid depletion after the fourth vaccine, concomitant with the first cycle of adjuvant TMZ. Responders showed a significant higher percentage of NK cells expressing GZMB at the second, fifth and sixth vaccination (P = 0.03, $P < 0.05$, compared to leukapheresis) (**Fig. 4E**). No expansion was assessed for GZMB expressing $CD8^+$ T cells neither in responders nor in non-responders (**Fig. 4F**).

Activated, IFN-γ positive $CD4^+$ T cells showed a late but significant expansion after the fourth vaccine ($P < 0.005$ vs. leukapheresis) (**Fig. 4G, Fig. S2C**). NK cells were also analyzed as $CD56^{dim}$ and $CD56^{bright}$ positive cells[14,15]. The $CD56^{dim}$ NK cell cytotoxic subset was significantly increased in responders and persisted at the end of the treatment (**Fig. S3**). The assessment of NK, $CD4^+$ and $CD8^+$ T cells in six patients treated with the standard Stupp regimen in the FluoGlio study[16] showed no significant changes in their frequency

after radio-chemotherapy (**Fig. S4**) suggesting that DC vaccinations induce NK cell activation. We also co-cultured PBLs from 8 patients with mature DCs loaded with tumor lysate. IFNγ production assessed by ELISA reflected values measured by flow cytometry in PBLs from responders, with a significant increase at third vaccination and decrease at fourth vaccination, after onset of adjuvant TMZ (**Fig. 4H**). No difference was found in non-responders (**Fig. 4I**). This observation suggests a potential direct involvement of specific anti-tumor effector cells producing IFNγ in response to tumor antigens presented by the dendritic cells.

The results indicate that NK cells are critical immune effectors during DC vaccination and suggest that they may synergize with $CD4^+$ T cells in a later phase, activating a clinically effective immune response.

$CD8^+$ T cells fail to develop a memory status and NK cells are intrinsically resistant to chemotherapy

To study further the effects of adjuvant TMZ on $CD8^+$ T cells, we examined by flow cytometry the generation of $CD8^+$ T cell central memory (TCM) and T effector memory (TEM) during and after treatment (**Fig. 5A, B**)[17]. In responders the significant increase of the $CD8^+$ TEM, frequency at second vaccination (P = 0.01) dropped permanently after adjuvant TMZ. We did not observe modulation of the TCM status. In non-responders we did not observe any significant expansion in the frequency of $CD8^+$ TEM and TCM during DC vaccines.

The poor contribution of $CD8^+$ T cells to anti-tumor responses was partly compensated by strong and long-lasting NK cell responses. In

order to understand this persistency, we investigated the potential involvement of the multidrug resistance protein ABCC3, based on our preclinical evidence that the efflux activity of ABCC3 confers chemoresistance to NK cells and protection from apoptotic cell death[18]. ABCC3 expression, investigated by real-time PCR, was significantly higher in responders (n = 8) than non-responders (n = 9; $P = 0.0005$) at the time of first vaccination after standard radio-chemotherapy (P = 0.02) and remained higher after concomitant vaccine and TMZ administration ($P < 0.01$) (**Fig. 5C**). Using flow cytometry, we found that ABCC3 was expressed by NK cells but not by $CD8^+$ T lymphocytes also in PBLs from 3 healthy volunteers (68.6 ± 16.8 vs. 1.2 ± 0.8, respectively; $P = 0.0002$, not shown). We also verified that at the time of leukapheresis (i.e. before radiochemotherapy), NK cells from responders displayed higher basal expression of ABCC3 than non-responders (28.7 ± 29.6% vs. 15.8 ± 17.6%, respectively; $P = 0.02$) (**Fig. 5D**). Responders showed a further increase of ABCC3 after DC vaccinations and concomitant TMZ. In $CD8^+$ T lymphocytes basal expression of ABCC3 was negligible (**Fig. 5E**) and in $CD4^+$ T lymphocytes quite low (**Fig. 5F**).

These results demonstrate that adjuvant TMZ limits the $CD8^+$ T cell response interfering with the memory status generation. ABCC3 plays a protective role against chemotherapy potentiating NK cell response and activity.

Tumor infiltrating NK cells are associated with a down-modulation of NK activating ligands expressed by tumor cells

To investigate whether an immune infiltration was detectable in specimens of patients who developed recurrence and underwent

second surgery, we characterized the tumor infiltrating lymphocytes (TILs). Specimens from Pt 8, 9, 10, 14 and 15 were analyzed for TILs by flow cytometry (**Fig. 6A** and **Fig. S5**) and by real time PCR for the expression of NK activating (MICA, MICB, ULBP1-3) or inhibiting (HLA-E) ligands and receptors (NKp30, NKp44, NKp46, NKp80, NKG2D) (**Fig. 6 B-E**). In three cases (Pts 9, 10, 15) NK cell infiltration reflected the peripheral NK cell response (V/B ratio>2.2). An impressive infiltration of NK cells was found in Pt 9 (responder) (**Fig. 6A**). The expression of NK ligands was totally lost, however, (**Fig. 6C**), a potential cause of the lack of tumor growth control. A reduction of NK activating ligands and receptors in second compared to first surgery specimen was also observed in Pt15, showing a massive NK cell infiltration (**Fig. 6B, C**). The second surgery of Pt10 was characterized by the removal of two different tumor specimens. The larger tumor mass showed the evolution from GBM to gliosarcoma with little immune infiltration, while the smaller fragment was highly infiltrated by NK cells and expressed higher levels of NK activating ligands and receptors compared to first surgery (**Fig 6 B-E**). $CD8^+$ and $CD4^+$ T cell infiltration for Pts 8, 9 and 15 are reported in **Fig. S5**. Pt 14 (non-responder) showed absence of immune cell infiltration, no expression of NK activating ligands and receptors, but significant up-regulation of HLA-E in both specimens.

These results indicate that peripheral and local NK cell response can be similar. However, the down-regulation of NK activating ligands can jeopardize the local anti-tumor activity of NK cells.

Discussion

Previously, we found that increased survival in patient with recurrent GBM treated by DC immunotherapy was primarily associated with tumor debulking and NK cell responses[19]. In the first stage of this phase II study, DENDR1, survival data of patients with primary GBM met the criteria for passage to the second stage of the Simon design. Data on OS were further analyzed using model 3 of the EORTC nomogram to predict survival in GBM patients taking into account MGMT methylation, Mini Mental Score Examination and WHO performance status. The actual OS in DENDR1 compared favorably with expected OS (13.0 months; 95% CI: 11.4-14.9). The expected OS was 66% at 12 months, and 23% at 24 months, $P = 0.004$[20].

While our study was running another study at our Institution, FluoGlio, enrolled patients with primary GBM that had surgery with intra-operatory use of fluorescein to help defining tumor borders[16]. Survival data in DENDR1 compare favorably with FluoGlio (median PFS and OS 10.5 and 20.1 months in DENDR1 and 7 and 12 months in FluoGlio).

Results also confirmed the safety of DC immunotherapy. One patient had fatal disseminated intravascular coagulation. However, the timing (last vaccination two months before death) and lack of data in the literature do not support the involvement of DC immunotherapy in this severe adverse event, while the natural history of malignant gliomas does include coagulation disorders[21].

DENDR1 demonstrates that the increase of NK cells in peripheral blood after DC vaccinations is strongly associated with prolonged

survival. Previous work showed that DC have a critical role in priming NK cells thanks to trans-presentation of interleukin (IL)-15[22,23]. NK cell collaboration with DC is critical to promote recruitment of effector $CD8^+$ T cells to the tumor microenvironment[24], an interaction that is critically dependent on IL-18 release[25,26]. However, recent data in murine models indicate that NK cells may also restrain spontaneous $CD8^+$ T cell priming through DC interaction mediated by the PD-1-PD-L1 checkpoint[27]. It is conceivable that such inhibition also took place in our patients, suggesting that combination with checkpoint inhibitors may boost the anti-tumor efficacy of DC immunotherapy.

Another constraint to the development of $CD8^+$ T cell antitumor activity and memory is likely due to their exquisite sensitivity to TMZ. We found that administration of adjuvant TMZ exerts negative effects on $CD8^+$ T cell activation and in particular on the generation of immune memory and central memory[17]. Long-term $CD8^+$ T cell responses were negligible not only in non-responders but also in responders, even if their pre-existing activation before RT/TMZ confirmed that GBM patients may exhibit tumor-specific $CD8^+$ T cells in peripheral blood[28].

Notably, results of DENDR1 confirmed our preclinical observation that the multidrug resistance protein ABCC3 is up-regulated in NK but not in $CD8^+$ T cells during TMZ treatment[18]. The expression and activity of this transporter were elements supporting chemoresistance in breast and non-small cell lung cancers but have not been fully appreciated as a tool of chemoresistance in immune cell[29,30]. As only a subset of patients benefits from chemo-immunotherapy combination, our hypothesis is that ABCC3 represents a predictive marker of

resistance of NK cells to TMZ forecasting a robust and long-lasting response associated to increased survival. This may be of critical relevance, as suggested by data in rodent models showing that intra-tumor but not systemic delivery of TMZ supports a strong $CD8^+$ T cell-based immune response[31]. In these experiments and in others showing lack of $CD8^+$ T cell activation, high dose TMZ was used (25-75 mg/kg)[11,31]. In the presence of low dose TMZ (2.5 mg/kg) or metronomic schedules, such activation was present[9,32,33]. Metronomic TMZ could be a viable option for combination with immunotherapy as it has been already used in primary and recurrent GBM with acceptable toxicity and some evidence of efficacy[9,34].

In conclusion, DENDR1 showed an encouraging gain in patient survival in the absence of major toxicity. Such gain appeared significantly dependent on NK cell activation. To complete such immune response with the contribution of $CD8^+$ T cells, the schedule of TMZ administration should be carefully re-evaluated.

Materials and methods

Clinical study

A 2-phase Simon design was used for the clinical study DENDR1 (EUDRACT N° 2008-005035-15)[12]. The primary goal was to evaluate progression free survival (PFS) rate 12 months after surgery (PFS12). Assuming as primary endpoint the percentage of PFS12 patients and an increase to 42% of the historical control rate of 27%[6], the alternative hypothesis will be rejected at the end of the first stage if the PFS12 rate will be less than 8/24 treated patients. Safety, feasibility and evidence of immune response are considered. The clinical protocol was approved by local and national regulatory authorities including Besta Ethical Committee, Istituto Superiore di Sanità (ISS) and AIFA (Italian Medicine Agency), and is sponsored by Istituto Neurologico Besta (see protocol as Supplemental File).

Population and treatment Protocol

Twenty-four patients with first diagnosis of GBM and no IDH1-2 mutations were enrolled after written informed consent, using the following inclusion criteria: histologically proven GBM, age ≥18 and ≤70 years, no multifocal or sub-ependymal diffusion of the tumor, residual tumor volume after surgery <10 ml, confirmed by postoperative Magnetic Resonance Imaging (MRI) assessment, dexamethasone daily dose ≤4 mg during the 2 days prior to leukapheresis, Karnofsky performance status (KPS) ≥70, non-necrotic tissue for lysate preparation and DC loading ≥1 gr and stored in liquid nitrogen, absence of past or current autoimmune disease. After surgery, patients underwent leukapheresis and radiochemotherapy

(RT/TMZ), according to the Stupp protocol[6]. DC were loaded with whole tumor lysate and produced under Good Manufacturing Practices (GMP) conditions[19,35,36]. On day 15 after surgery, leukapheresis and basal clinical, radiological and immune testing were performed. Delayed-Type Hypersensitivity (DTH) skin reactions, injecting Ag purified tuberculin as control and 10 mg of inactivated tumor lysate, were tested before and after vaccinations 1-4. The first 4 vaccinations with tumor lysate loaded DC were performed every two weeks, from week 9 to 15. After the fourth vaccine, MRI was performed. Vaccinations 5 and 6 were spaced one month (week 19 and 23, respectively). The last vaccine dose (the 7th) was on week 31. At each vaccine injection, clinical and immune monitoring was performed. From the end of immunotherapy on, MRI, clinical and immune monitoring were continued every 2 months. The 1st, 5th, 6th and 7th vaccines contained 10 million DC; the 2nd, 3rd and 4th vaccines 5 million DC. Adjuvant TMZ started immediately after 3rd vaccination and continued for 6 cycles (**Fig 1A**).

MRI and response evaluation

Patients underwent conventional contrast enhanced MRI (see Supplementary Data for detailed radiological protocol) within two days after surgery, within two days before the first vaccination, every two months, or in case of clinical worsening. Tumor volumes were determined on the 3D post gadolinium T1 weighted images by manually outlining the enhancing portion of the lesion in MRIcro (http://www.mricro.com). To calculate the total enhancing volume of the tumor, the number of enhancing voxels was multiplied by the

voxel size. Disease progression was defined according to RANO criteria[37].

Immune monitoring

Immune monitoring was performed on the whole blood of each patient before, during and after DC vaccinations. The immune responses were assessed before the treatment, after each vaccination and at the end of the treatment until tumor recurrence. Eight patients were re-operated (Pt 2, 8, 9, 10, 14, 15, 16, 24). No adequate/sufficient material was obtained from surgery of Pt 2 and 17; Pt 24 underwent surgery in another institute. Tumor infiltrating immune cells were isolated by tumor specimens obtained from Pt 8, 9, 10, 14 and 15 using human Tumor Dissociation Kit in combination with GentleMACS (Miltenyi Biotec). Antibodies, staining for effector activation, memory status formation and real time PCR protocols are reported in Supplementary Data.

Statistical analyses

The ratio of the mean of vaccinations (2nd to 7th)/baseline values (V/B ratio) of absolute count and frequency of NK cells, $CD8^+$ and $CD4^+$ T cells for each patient was calculated, and the median of all of the observations was used as the cut off value to separate patients into the "low" or "high" groups. The threshold able to separate patients with "low" or "high" V/B ratio and having the best sensitivity and specificity, was defined using Receiver Operating Characteristic (ROC) curves. PFS was calculated from first surgery until disease progression and death/last follow-up, if censored. Overall Survival (OS) was calculated from surgery to death due to any cause or last

follow-up (censored). Kaplan-Meier analysis was used to estimate PFS and OS. The log rank test assessed differences in progression or survival in patients with different immunological or clinical parameters.

Figures

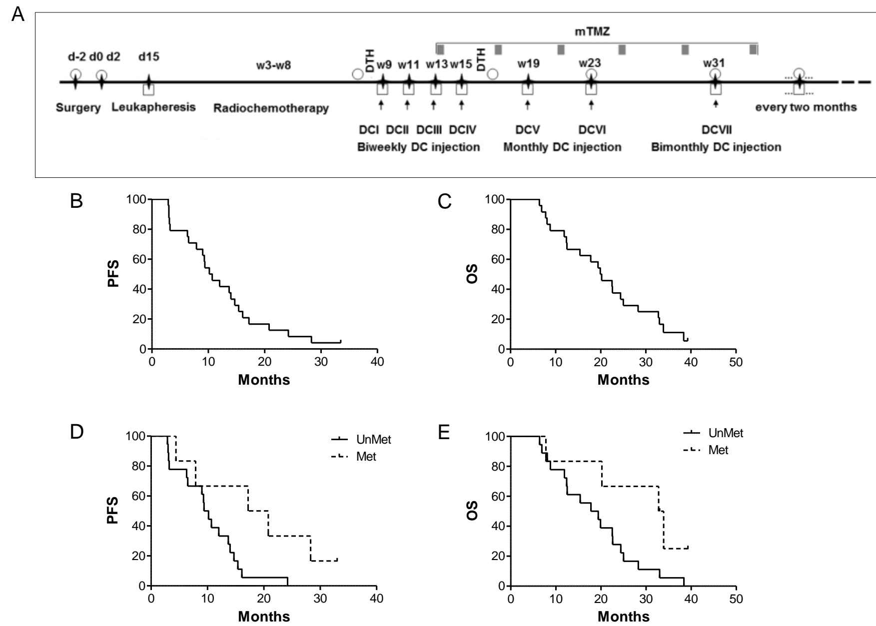

Figure 1 Survival analysis of 24 GBM patients treated by DC immunotherapy. (A) Treatment protocol showing the timing before, during and after the DC administrations. D = day, W = week, White circle = MRI, Black diamond: clinical monitoring, White square = immune monitoring except DTH, DCn = vaccine number, Gray rectangle = maintenance TMZ (5 days/28 for 6 cycles). (B) Kaplan Meier curves showing progression free survival (PFS) and (C) overall survival (OS) of the 24 patients enrolled in the first stage of DENDR1. Kaplan Meier curves showing PFS (D) and OS (E) of patients, with methylated or unmethylated MGMT promoter in the tumor.

Patient	Age/Gender	KPS post-surgery	Steroids at 1st vaccination (mg)	No. of vaccinations	No. of TMZ cycles	MGMT (Met≥0.1)	NK cell V/B ratio	PFS (mts)	OS (mts)	Treatment after IT
1	55/F	100	0.00	7	6	U (0.07)	0.7	13.7	22.5	No treatment
2	62/F	100	1.25	7	5	U (0.01)	0.7	12.0	24.4	Bevacizumab
3	66/M	80	0.00	7	6	U (0.04)	2.8	15.4	15.4	No treatment
4	70/F	80	0.00	7	6	U (0.00)	1.3	14.7	17.8	Bevacizumab
5	49/M	90	0.00	7	6	U (0.00)	1.7	10.2	12.5	No treatment
6	65/F	70	4.00	7	6	M (0.71)	3.0	20.8	33.9	Metronomic TMZ
7	60/M	80	0.00	7	6	U (0.01)	1.7	9.3	25.0	Bevacizumab
8	58/M	100	2.00	7	6	U (0.00)	2.2	9.4	22.6	Bevacizumab
9	50/M	90	0.00	7	6	U (0.00)	5.0	16.1	33.0	TMZ, Cyber, PCV
10	48/M	80	2.00	3	2	M (2.83)	4.0	4.4	7.8	Bevacizumab
11	23/F	90	0.00	4	1	U (0.003)	0.7	3.1	6.4	No treatment
12	44/M	70	0.00	7	6	U (0.03)	2.8	24.2	38.4	PCV
13	55/M	70	4.00	6	6	U (0.00)	1.9	10.7	19.4	No treatment
14	62/M	80	0.00	7	6	M (0.46)	0.7	7.9	20.2	Bevacizumb
15	36/M	100	2.00	5	2	U (0.00)	3.2	2.9	19.9	RT
16	70/M	80	4.00	7	6	M (1.50)	2.9	17.2	32.8	CCNU
17	56/F	80	4.00	4	1	U (0.00)	1.5	3.0	11.9	Bevacizumab
18	49/M	80	4.00	4	2	U (0.00)	2.1	6.3	8.8	No treatment
19	56/M	70	4.00	4	1	U (0.02)	0.6	3.2	6.9	No treatment
20	48/M	70	4.00	5	6	U (0.00)	0.6	9.0	12.4	CCNU
21	53/F	90	0.00	7	5	M (0.47)	5.0	28.3	>39.3	TMZ,PCV
22	63/M	90	4.00	5	3	U (0.02)	3.4	6.5	8.1	Bevacizumab
23	45/M	90	0.00	7	6	M (0.74)	2.7	>33.0	>33.0	No treatment
24	55/F	80	0.00	7	6	U (0.00)	3.2	14.0	28.3	Fotemustine

Table 1 Patients characteristics.

Abbreviations. KPS: Karnofsky performance status; TMZ: temozolomide; MGMT: O6-methylguanine-DNA-methyltransferase; PFS: progression free survival; OS: overall survival; PCV: Procarbazine, Lomustine, and Vincristine.

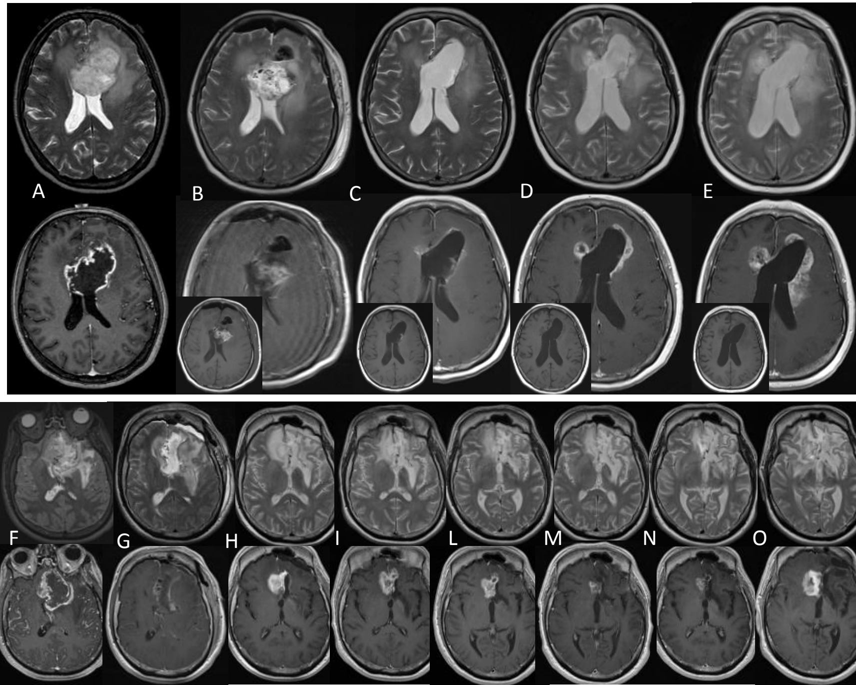

Figure 2 Exemplificative MRI. Patient 17. Top, T2 weighted images (w.i.); bottom, contrast enhanced T1 w.i. (small box, pre-contrast T1 w.i.). (A) Pre-surgery, Dec 5, 2012 (necrotic lesion, GBM). (B). Post-surgery: Dec 6, 2012 (blood presence). (C-E) Immunotherapy: Jan, Mar and May 2013 (progressively enhancing lesion). Patient 12. Top, T2 w.i., bottom, contrast-enhanced T1 w.i. (F) Pre-surgery May 14, 2012 (necrotic lesion, GBM). (G) Post-surgery May 18, 2012 (scarce blood). (H-O) Immunotherapy: H-L (Aug 2012, Nov 2012, Jan 2013) show enhancing lesion. (M) subsequently reduced (M, Mar 2013) suggesting pseudoprogression. (N) subsequent stabilization (May 2014). (O) and disease progression (July 2014).

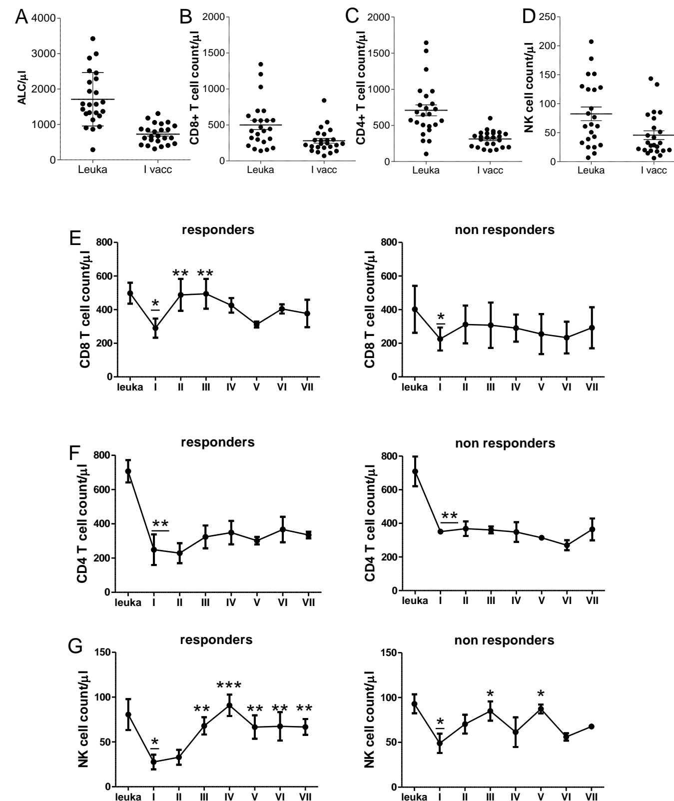

Figure 3 Radiotherapy and chemotherapy impact on CD8$^+$, CD4$^+$ T and NK cell counts. (A) Absolute lymphocyte counts in the peripheral blood of patients before (leukapheresis = leuka) and after (first vaccination = I vacc) RT-TMZ. (B-D) CD8$^+$, CD4$^+$ T and NK cell absolute counts before and after RT-TMZ. (E-G) Time course of CD8$^+$ T cell count, CD4$^+$ T cell count and NK cell count measured by flow cytometry of responder (left, n = 9) and non-responder (right, n = 15) patients over the treatment (*P <0.01, **P < 0.005, ***P < 0.0005 vs. 1st vaccine; underlined asterisk * vs. leukapheresis). Data are presented as mean ± SEM.

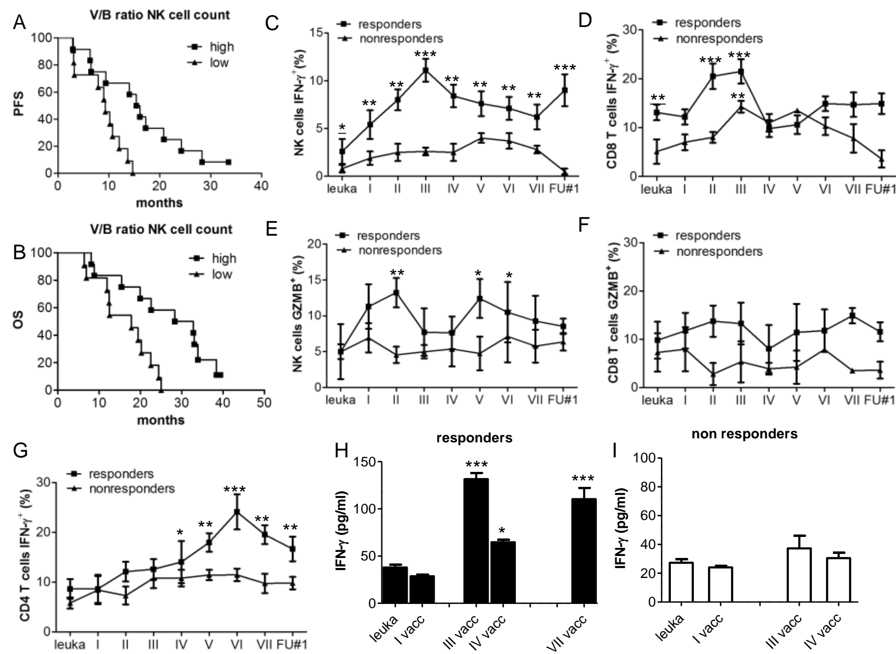

Figure 4 DC vaccination induces a significant activation of NK cells correlated with an increased survival of patients. (A, B) Kaplan-Meier analysis of the correlation between V/B ratio of NK cell counts with (A) PFS and OS (B). Median PFS of patients with high V/B ratio (> 2.2, n = 11) vs. low (≤2.2, n = 13): 16.1 months vs. 9.3 months (P = 0.0025); median OS: 32.8 months vs. 17.8 months (P = 0.0039). (C-D) Time course of frequency of NK cells (C), $CD8^+$ T cells (D) expressing IFN-γ measured by flow cytometry (*P < 0.01, **P < 0.005, ***P < 0.0005 vs. leukapheresis; underlined asterisk responders vs. non-responders). Representative dot plots are shown in Fig. S.2. (E, F) Time course of frequency of NK cells (E), $CD8^+$ T cells $CD4^+$ T cells (F) expressing GZMB measured by flow cytometry (G) Time course of $CD4^+$ T cells expressing IFN-γ measured by flow cytometry. Data are presented as mean ± SEM. (H-I) Time course of IFN-γ secretion by PBLs from (H) 4 responders and (I) 4 non-

responders, co-cultured for 5 days in the presence of autologous loaded mature DC.

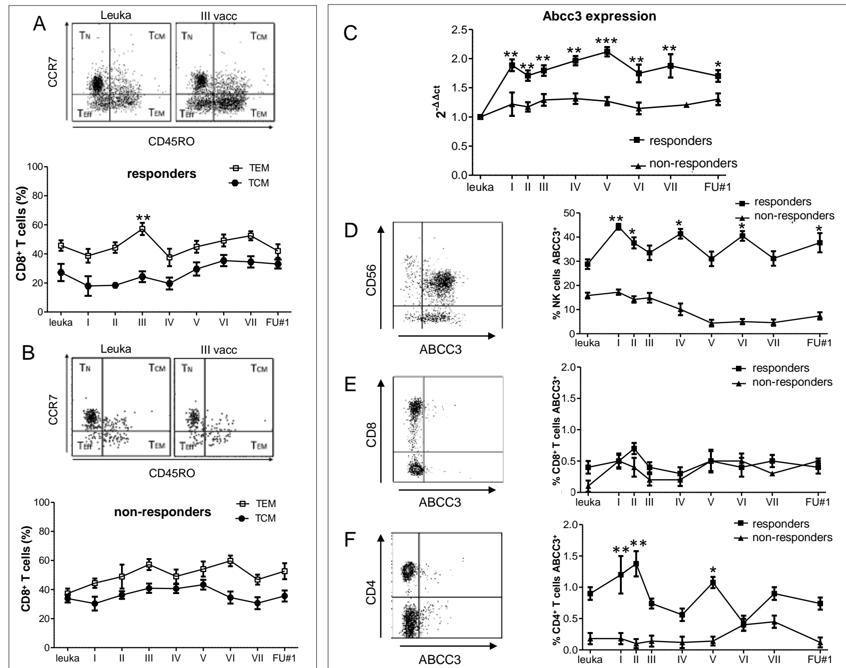

Figure 5 NK cells, but not $CD8^+$ T cells express and are resistant to chemotherapy. (A-B) Time course of $CD8^+$ T effector memory and central memory cells evaluated by flow cytometry on PBMCs from 5 responders (A) and 6 non-responders (B) (**P < 0.005 vs. leukapheresis). (C) Time course of Abcc3 expression assessed by real-time PCR on PBLs of 17 patients (8 responders and 9 non-responders). (D-F) Time course of NK cell, $CD8^+$ T cell and $CD4^+$ T cell frequency expressing ABCC3, assessed by flow cytometry (*P < 0.01, **P < 0.005, ***P < 0.0005 vs. leukapheresis, P = 0.02 *underlined asterisk responders vs. non-responders). Data are presented as mean ± SEM.

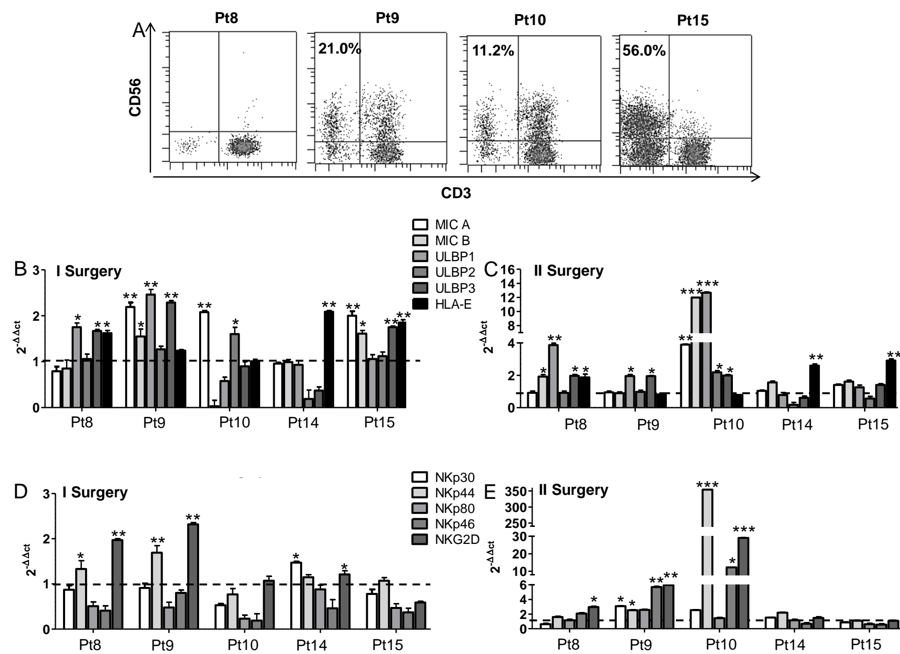

Figure 6 Tumor infiltrating NK cell activity was influenced by the expression of activating or inhibiting NK ligands and receptors. (A) Dot plots showing the percentage of NK cells infiltrating tumor mass of four patients as evaluated by flow cytometry. (B-E) Bar graphs showing the relative expression of activating (MICA, MICB, ULBP1-3) or inhibiting (HLA-E) ligands and receptors (NKp30, NKp44, NKp46, NKp80, NKG2D) in specimens from first and second surgery of five patients (four non-responders – Pts 8, 10, 14, 15 – and one responder – Pt9), evaluated by real time PCR. The relative expression of ligands and receptors was compared with that detected in normal brain tissue (dotted line).

Supplementary Material

For supplementary material:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5889286/

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Chapter 3

Expansion of effector and memory T cells is associated with increased survival in recurrent glioblastomas treated with dendritic cell immunotherapy

Marica Eoli*, **Cristina Corbetta***, Elena Anghileri*, Natalia Di Ianni, Micaela Milani, Rosina Paterra, Simona Frigerio, Sara Nava, Daniela Lisini, Sara Pessina, Maria Tardini, Paolo Ferroli, Francesco DiMeco, Valeria Cuccarini, Maria Grazia Bruzzone, Carlo Antozzi, Bianca Pollo, Gaetano Finocchiaro, Serena Pellegatta

*** These authors contributed equally to this work.**

SUBMITTED

Abstract

Background: The efficacy of Dendritic Cell (DC) immunotherapy as a single therapeutic modality for the treatment of glioblastoma (GBM) patients is limited. In this study, we evaluated the immune-mediated effects of DC immunotherapy combined with temozolomide (TMZ) or tetanus toxoid (TT) in recurrent GBM patients.

Methods: In the phase I-II clinical study DENDR2, twelve patients were treated with five DC vaccinations combined with a dose-dense TMZ. The subsequent pilot study named Variant (V)-DENDR2 included eight patients: the vaccine site was preconditioned with TT 24 hours before DC vaccination and TMZ was avoided. As a survival endpoint for these studies we considered overall survival 9 months (OS9) after second surgery. Patients were analyzed for the generation of effector, memory, and T helper immune response.

Results: In DENDR2 four out of twelve patients reached OS9, but all failed to show an immunological response. In V-DENDR2 five out of eight patients reached OS9, and one patient is still alive (OS>24 mos). A robust $CD8^+$ T cell activation and a memory T cell formation were observed in V-DENDR2 OS>9. Only in these patients, the vaccine-specific $CD4^+$ T cell activation was paralleled by an increase in TT-induced $CD4^+$ memory T cells. Only V-DENDR2 patients showed a formation of a nodule at DC injection site infiltrated by CCL3 expressing-$CD4^+$ T cells.

Conclusions: TT pre-conditioning of the vaccine site and lack of TMZ contribute to the efficacy of DC immunotherapy by inducing a strong effector response, helper and memory T cell generation.

Introduction

Recurrence of glioblastoma (GBM), the most malignant primary brain tumor, does not have a standard treatment and is associated with a poor prognosis: median survival from recurrence is about 9 months[1,2]. Immunotherapy has accomplished important prognostic improvements in different cancers and particularly in melanomas, mostly due to the treatment with checkpoint inhibitors[3]. However, in GBM patients evidence of meaningful clinical responses to checkpoint inhibitors is presently lacking. Scarce and rare infiltration of T lymphocytes in the tumor, low mutational load and the presence of a strong immune suppressive microenvironment can support resistance to checkpoint inhibitors and could limit their success in GBM immunotherapy[4].

Dendritic cells (DCs), powerful antigen presenting cells, are an important tool for cancer immunotherapy[5]. The efficacy of DC immunotherapy as single therapeutic modality, however, is limited and rarely curative. This condition has generated considerable interest in combinatorial strategies.

Notably, some chemotherapeutic drugs may cause an immunogenic cell death, leading to some synergy with immunotherapy. In case of temozolomide (TMZ), however, preclinical data and our own experience indicate that specific chemotherapy can impair the anti-tumor activity of $CD8^+$ T cells[6–9].

The efficiency of DC migration from the injection site to the lymph nodes (LNs) can represent another critical aspect influencing the success of immunotherapy. It has been observed that less than 4-5% of injected DCs can reach the LNs[10]. Recent data from Mitchell and colleagues indicated that pre-conditioning the vaccine site with the

recall antigen tetanus/diphtheria toxoid (Td) can induce a specific inflammatory immune response mediated by Td-specific $CD4^+$ T cells and the production of CCL3, improving LN homing of DCs and consequently, the efficacy of tumor-antigen-specific DCs[11].

Tetanus toxoid (TT) alone was recently used as an adjuvant in phase I clinical study in combination with DC immunotherapy and IL-2 administration. In a small fraction of patients, a specific immune activation was associated to clinical benefit without side effects and toxicity[12].

Here, we report and compare the clinical and immunological data of DENDR2, a clinical study in recurrent GBM patients, in which DCs were combined with a dose-dense TMZ, with Variant (V)-DENDR2 study, in which recurrent GBM patients were treated with DCs after pre-conditioning of the injection site with tetanus toxoid recall in the absence of TMZ.

Results

Patient treatment and survival

Two cohorts of patients were considered: patients with recurrent GBM enrolled in the DENDR2 (EUDRACT No 2008-005038-62, n=12) clinical study, and patients with recurrent GBM treated on a compassionate basis with DC immunotherapy concomitant with TT in the absence of TMZ (n=8) named (V)-DENDR2. We considered OS9 as a relevant survival endpoint based on an extensive analysis of GBM patients treated at our Institution[1] and on recent data from a phase III study in recurrent GBM[2].

In DENDR2 recurrent GBM patients were treated with five injections of DCs and TMZ with a dose-dense schedule (TMZ 3 weeks on, 1 week off for three cycles)[13]. Sixteen patients were enrolled, but only fifteen were evaluable for the efficacy endpoints (i.e. they received at least three doses of DC vaccination). We restricted the patient analysis to the patients with IDH1 wild-type GBM (n=12) as its value for survival analysis was not appreciated when the DENDR2 protocol was established. The schedule of the treatment and clinical data of the 20 patients (DENDR2 + V-DENDR2) are summarized in **Fig. 1** and **Table 1**. The median interval between first and last surgery was 14.0 months (95%CI 11.2-25.6). Four patients completed all scheduled vaccinations, two patients discontinued treatment after four vaccinations and six after three. Five patients completed the TMZ schedule, five could be treated with two out three cycles and two with one cycle only. Before surgery for recurrence, seven completed the Stupp protocol[14]. The median OS of DENDR2 patients was 7.4 months (95%CI 5.2-9.31) and OS9 was 33.3% (**Fig. 2A**). The median

interval between last surgery and the first vaccine was 1.6 months (95%CI 1.4-1.78). All patients experienced death during the follow-up due to tumor progression.

In four DENDR2 patients, disease progression occurred before first vaccination. Hypermethylation of the MGMT promoter was detected in four out of the eleven patients with enough DNA available for the analysis: two of them were long-term survivors. Two patients did not receive active treatment after progression disease (Pts 16 and 17); other treatments included bevacizumab (four cases), chemotherapy (eight cases), chemotherapy and radiotherapy (one case) (**Table S1**).

In the V-DENDR2 study, five patients completed all scheduled vaccinations, one discontinued treatment after four vaccinations and two after three vaccinations. Patient V-1 and V-4 suspended the treatment after the third vaccination due to clinical worsening and restarted the vaccination after five and two months, respectively. Three patients were treated also with 2 cycles of PCV (procarbazine, CCNU and vincristine), the others received no further treatment (**Table S1**). After a median follow-up of 9.2 months, one of eight patients was alive, six died for tumor progression and one for pulmonary embolism. Hypermethylation of the MGMT promoter was detected in 4/8 patients. Two of them survived longer than 9 months. Mutations in the IDH1 gene were not detected in any patient, confirming that all patients suffered from primary GBM. The median OS was 9.2 (95%CI 5.2-9.31) months and OS9 was 62.5% (**Fig. 2A** and **Table 1**).

Exemplificative MRI of one V-DENDR2 is displayed in **Fig. 2B-F.**

Treatments as planned in both protocols (DENDR2 and V-DENDR2) were safe and well tolerated

As reported in **Table S2**, in DENDR2 adverse events (AE) were mostly transient neurological worsening (IT-related), hematological toxicities (TMZ-related) or surgical-linked; no site-injection AE were reported. In V-DENDR2 we observed some AE linked to the pre-existing neurological symptoms related to the tumor lesion and so were peculiar to each patient (**Table S2**). Five patients showed neurological worsening after the third vaccination, one after the last vaccination and two showed no symptoms. Moreover, three cases of seizures, one case of headache and confusion (V-2) and one case of pneumonia (V-3) related to the tumor progression, were also reported. Skin reactions at the injection site were also reported. They are characterized by skin redness and/or thickening and, at the same time they constitute both an adverse dermatological effect and an indirect manifestation of vaccine immune response. Three patients (V-1, V-2 and V-5) had a weak skin reaction, on the contrary, three patients (V-3, V-4 and V-6) showed a great skin reaction associated with inflammation at the injection site. Non-serious skin reactions were recorded at the injection site after TT administration.

Pre-conditioning of the vaccine site and absence of temozolomide contributed to $CD8^+$ and $CD4^+$ T cell activation in V-DENDR2 long-term survivors (OS>9)

We assessed patient immune responses considering the count and frequency of peripheral blood lymphocytes (PBLs) before the treatment, at each vaccination (pre- and post-conditioning with TT, in the case of V-DENDR2) and after immunotherapy. We previously

demonstrated that RT-TMZ treatment impacted on absolute lymphocyte count (ALC) in DENDR1 patients, inducing significant lymphopenia[9]. In DENDR2 patients we observed a basal ALC > 1000 cells per ml peripheral blood (1504.4 /ml ± 792.0/ml, mean ± SD) at leukapheresis in 9/10 patients, that decreased after TMZ treatment, although not significantly (1096.5/ml ± 530.4/ml, mean ± SD) (**Fig. 3A**). In V-DENDR2, ALCs were 1704.6/ml ± 666.0/ml at leukapheresis and went to 1390.6/ml ± 573.3/ml the day after the first vaccination (**Fig. 3B**). The absolute count of both $CD8^+$ and $CD4^+$ T cells increased significantly after the second (P=0.02), third (P<0.05), and fourth vaccination (P=0.04) only in V-DENDR2 long-term survivors (OS>9) (Fisher's exact test P=0.01) (**Fig. 3C, D**). Neither V-DENDR2 short-term survivors (OS≤9) nor DENDR2 patients showed a positive modulation of T cell counts (**Fig. 2E, F**).
The activation status of $CD8^+$ and $CD4^+$ T cells was investigated by analyzing their IFNγ expression. V-DENDR2 and DENDR2 long-term survivor patients were compared with short-term survivors. A significant increase of $CD8^+$ T cells expressing IFNγ was observed in V-DENDR2 long-term survivors at second vaccination compared to the baseline (19.8 ± 5.5 vs. 11.8 ± 8.6, P<0.05) (**Fig. 3G** and **H**, Supplementary **Fig. S1A, B**). Kinetics were highly dynamic with a contraction phase between the third and fourth vaccination and a rapid second increase at fifth vaccination and follow-up (19.9 ± 2.6 and 23.6 ± 3.2 respectively, P<0.05 compared to the baseline). A similar, although weaker, response was observed for $CD4^+$ T cells (Supplementary **Fig. S1C, D**). A significant NK cell response was

detected only in these patients and at earlier time points (Supplementary **Fig. S1E, F**).
In V-DENDR2 short-term survivors and in all of the DENDR2 patients, T cells did not show any activation sharing a similar response kinetics during the treatment (**Fig. 3D, E,** and **F**).

$CD8^+$ T cells differentiated into long-lasting memory T cells retaining the ability to express IFNγ

To characterize the transition of effector T cells towards the memory, we defined the antigen-experienced $CD8^+$ and $CD4^+$ T cells based on their expression of the antigen-experienced, effector T cell marker KLRG1[15] (**Fig. 4A-D**, Supplementary **Fig. 2 A, C**). $CD8^+$ T effector cells co-expressing high levels of KLRG1 and IFNγ showed a significant expansion and contraction phase only in V-DENDR2 long-term survivors (**Fig. 4C**). A significant increase from the baseline was found at the second vaccination (6.4 ± 3.5% vs 0.9±0.8%, respectively; $P<0.005$). A rapid reduction started during the contraction phase (4.7 ± 2.7% vs second vaccination) and the memory phase at the follow-up (2.1 ± 1.8% vs second vaccination; $P<0.001$).
Effector to memory transition of $CD4^+$ T cells was less robust than the $CD8^+$ T cell response, decreasing slowly over time (Supplementary **Fig. S2A, B**). Notably, in short-term survivors, $CD8^+$ $IFN\gamma^+$ T cells retained high expression of KLRG1, precluding the formation of long-lasting T cell memory (**Fig. 4C**). No significant increase of $KLRG1^+$ T cells was detected in DENDR2 patients, indicating the lack of active and functional effector T cells (**Fig. 4D**). A molecular evaluation performed by real time-PCR revealed a predominant expression of EOMES and ID3 genes, indicating an enrichment for T central

memory (Tcm) exclusively in V-DENDR2 long-term survivors during the contraction phase (at the time of the biopsy and fourth vaccination) (**Fig. S2C**). At the same time points, short-term survivors showed an increased expression of the T effector memory (Tem)-associated genes T-BET and/or PRDM1 (**Fig. S2C**). No significant differences in memory composition were found in DENDR2 patients (**Fig. S2D**).

Tetanus toxoid recall induced expansion of TT-specific $CD4^+$ T cell response supporting the vaccine-specific T cell response

To evaluate the presence of vaccine-specific or TT recall induced-$CD4^+$ T helper memory cells, first we analyzed the memory T cell subsets identifying the $CD45RA^-$ $CCR7^-$ as Tem and $CD45RA^-$ $CCR7^+$ as Tcm. A $CD4^+$ $CD38^{+/high}$ $IFN\gamma^+$ T cell subset (mainly $CCR7^-$, consistent with vaccine-induced Tem), was found expanded in V-DENDR2 long-term survivors (**Fig. 4E**, Supplementary **Fig. S2E**), and a $CD4^+$ $CD38^{+/low}$ $IFN\gamma^+$ T cell response (predominantly $CCR7^+$, consistent with Tcm), displayed similar kinetics (**Fig. 4E**). Both these subsets were absent at the baseline (first vaccination), displayed a rapid activation after the second vaccination (fourth vaccination vs baseline: $CD38^{+/high}$ cells: 3.4±0.5% vs 0.4±0.06%, P<0.005; $CD38^{+/low}$ cells: 2.8 ± 0.4% vs 0.7 ± 0.3%, P<0.01), and retained this phenotype at the follow-up ($CD38^{+/high}$ cells: 2.8 ± 0.3% vs 0.4 ± 0.06%, P<0.005; $CD38^{+/low}$ cells: 2.5 ± 0.7% vs 0.7 ± 0.3%, P<0.001) .

In V-DENDR2 short-term survivors, these two subsets of $CD4^+$ T cells were absent at the baseline (**Fig. 4F**). Only the $CD38^{+/high}$ T cells significantly increased at the third vaccination (2.1 ± 0.5% vs 0.3 ±

0.04%), and returned rapidly to baseline values. These subsets were not detectable in DENDR2 patients (Supplementary **Fig. S2F** and **G**). The $CD4^+$ Tcm cells also expressed high levels of CD127, the receptor of IL-7 involved in memory T cell survival and persistence, and low levels of CD25 in V-DENDR2 long-term survivors only (**Fig. 4G**). Overall, the expansion of vaccine-specific, activated memory T cells was accompanied by an increase of a bystander $CD4^+$ T helper memory cells defined as $CD38^{+/low}$ and CD127+/CD25low. Modulation of $CD4^+$ T helper memory cells was absent in both V-DENDR2 short-term survivors and DENDR2 patients (**Fig. 4G, H**).

Thickenings formed at the tetanus toxoid injection site are infiltrated by CCL3 - expressing $CD4^+$ T cells

To confirm the contribution of the TT pre-conditioning in enhancing the efficacy of the DC vaccinations, we monitored the appearance of a local reaction during the treatment.

None of the 12 DENDR2 patients, showed a local reaction. Only in V-DENDR2 patients we observed a formation of granulomas at the DC injection site, appearing as localized thickening with different sizes, that we removed by a skin biopsy after the third vaccination. As controls, we used the thickening that appeared at the DC injection site in a minority of patients enrolled in the DENDR1 study[9]. Staining for CD4 and CCL3 was performed on adjacent sections of the skin biopsies derived from two V-DENDR2 long-term survivors and two DENDR1 responders, as controls (**Fig. 5 A-D**). The V-DENDR2 skin biopsies were characterized by a dense dermal infiltration of CD4 and CCL3 positive cells (**Fig. 5 A, B**). In DENDR1 patients the CD4

positive cells were preferentially distributed near the upper layer of the dermis, and double positive cells were not found (**Fig. 5 C, D**).

Discussion

The delineation of an optimal combination strategy to improve immunotherapeutic approaches is still an ongoing challenge. A potential synergy between immunotherapy and chemotherapy has been reported due to the influence of some chemotherapeutic agents on tumor-specific immune responses, either by inducing immunogenic cell death of tumor cells or by modulating key cells for immune suppression or activation[16–18]. In two clinical studies on newly diagnosed or recurrent GBM that have been active at our Institution, vaccinations with DCs loaded with whole tumor lysate were combined with chemotherapy and TMZ administered as a potential adjuvant.

We obtained encouraging results in a fraction of the newly diagnosed GBM patients (45%) associated with a rise of active NK cells in peripheral blood: however, the contribution of $CD8^+$ T cells to antitumor activity and the memory status generation were likely jeopardized by TMZ combination[9].

Thus, our experience with DC immunotherapy indicated that systemic administration of TMZ can limit the anti-tumor immune response and, in particular, the action of $CD8^+$ T cells and their long-lasting response.

Recent experiments sustained that intraperitoneal delivery of BCNU does not synergize with immunotherapy[19] while the local delivery of BCNU “wafers” into the tumor cavity significantly enhanced the antitumor activity of checkpoint inhibitors, supporting the idea that the systemic administration of chemotherapy is not effective as immunotherapy adjuvant.

The present study compares two different strategies of DC immunotherapy for recurrent GBM patients. In the clinical study DENDR2, where DC vaccinations were combined with dose-intense TMZ, we were unable to detect any significant immune response activation and survival advantage. Only four DENDR2 patients (two with methylated -Pts 11 and 25- and two with unmethylated -Pts 19 and 28- MGMT promoter), survived more than 9 months, but correlations with peripheral immune activation were weak in two patients (Pt 25 and 28 showing a minor increase of NK cell frequency between the second and the fourth vaccinations) and absent in the others.

This data corroborates the prior clinical study on the total absence of $CD8^+$ T cell activation. However, these patients also failed to show the marked activation of NK cells observed in DENDR1 patients[9]. We hypothesize that a prolonged stimulation and exposure of NK cells to TMZ may cause their dysfunction and exhaustion. Indeed, NK cells are susceptible to become exhausted and unable to produce IFNγ and exert cytotoxic activity[20]. Furthermore, NK cells can acquire an exhausted phenotype during tumor progression, through the action of the checkpoint receptor TIGIT[21].

Based on clinical and immunological negative data from the twelve DENDR2 patients, we set-up a pilot study named V-DENDR2 where TMZ was avoided.

We also considered the ability of DCs to migrate to lymph nodes (LNs) as another critical aspect impinging on the efficacy of immunotherapy. Recently, Mitchell and colleagues have demonstrated that anti-tumor activity associated with DC vaccinations can be

increased by administering TT as a pre-conditioning step[11]. As virtually all people have been vaccinated against tetanus, the recall antigen Td can attract CD4^{+} T cells locally that in turn release the CCL3 chemokine, up-regulating the expression of CCL21. This chemokine improved DC homing to the LNs and was associated with evidence of a significant clinical advantage in a murine model of GBM and in a limited number of patients[11].

Accordingly, in V-DENDR2 eight recurrent GBM patients received TT the day before DC injection and no TMZ. Increased survival and specific effector and helper immune responses were found in 5 of these patients who survived longer than 9 months ("long-term survivors"). In two of them (V-1 and V-4), DC immunotherapy was temporarily discontinued, suggesting that their clinical symptoms were due to tumoral inflammation rather than progression.

We also suggest that the absence of TMZ allowed the activation of CD8^{+} T cells and the memory formation evaluated as loss of KLRG1 expression[15], as found in five V-DENDR2 patients and never in DENDR2 patients.

Based on the evidence that CD4^{+} T cells respond to the TT[22], we examined the expansion of TT-specific CD4^{+}T cells, looking for the expression of CD38 and CD127 markers[23].

The increased frequency of CD4^{+} T helper cells that are required to help CD8^{+} T cell responses[24] was associated with clinical advantage in five patients, who all survived longer than 9 months.

Also, a dense infiltration of cells positive for CD4 and CCL3, was visible in skin biopsies obtained after the sequence of TT conditioning

and DC injection, in good agreement with data from Mitchell and colleagues[11].

These findings encourage larger studies on GBM patients in the recurrent setting using DC after pre-conditioning of the injection site (https://clinicaltrials.gov/ct2/results?cond=glioma&term=tetanus&cntry=&state=&city=&dist=). Result confirmation may open the way to further studies attempting to increase DC responses and other immunotherapy approaches by increasing neoantigen presentation by local chemotherapy[19], radiotherapy[25] and possibly high intensity focused ultrasounds[26].

Materials and Methods

Clinical study

DENDR2. DENDR2 study (EUDRACT No 2008-005038-62) was a phase I/II, two-stage Simon design, non-randomized clinical study in which patients with recurrent GBM were treated with immunotherapy with DCs loaded with autologous tumor lysate in combination with dose-dense TMZ. Safety, feasibility, and evidence of immune response were considered. The clinical protocol was approved by local and national regulatory authorities including Besta Ethical Committee, Istituto Superiore di Sanità (ISS) and AIFA (Italian Medicine Agency), and was sponsored by Fondazione IRCCS Istituto Neurologico Carlo Besta.

V-DENDR2. A pilot study named Variant (V)-DENDR2 satisfying the DENDR2 inclusion criteria, including DC vaccination combined with TT pre-conditioning, was performed on a compassionate basis on 8 patients with recurrent GBM.

Population and treatment protocol

Sixteen patients with diagnosis of recurrent GBM were enrolled in DENDR2 after written informed consent inclusion criteria that were the following: histologically proven GBM, age ≥18 and ≤70 years, no multifocal or sub-ependymal diffusion of the tumor, residual tumor volume after surgery <10 ml, assessed postoperative MRI, dexamethasone daily dose ≤4 mg during the 2 days prior to leukapheresis, Karnofsky Performance Status (KPS) ≥70, availability of 0.8-1 g tissue for lysate preparation stored at -80°C, absence of past or current autoimmune disease. After surgery, patients underwent

leukapheresis. DCs were loaded with whole tumor lysate and stored following Good Manufacturing Practice (GMP) conditions[27,28]. The first three vaccinations with DC were performed every two weeks (weeks 6 to 10). The fourth and fifth vaccinations were spaced one month (week 14 and 18, respectively). At each vaccine injection, clinical and immune monitoring was performed. DCI contained 20 million cells; DCII-IV contained 10 million cells; DCV 5 million cells. TMZ was administered for three cycles according to the schedule 21/28[13], before DCI, DCIV and DCV. At each vaccine injection, clinical and immune monitoring were performed.

In the pilot study V-DENDR2, eight patients were enrolled after written informed consent and treated with the above doses of DC vaccinations, pre-conditioning the vaccine site with the recall antigen (TT) (40 U.I intradermally injected) the day before each DC vaccination. No TMZ was administered. Clinical, immunological and radiological evaluations were performed with the same schedule as DENDR2. A skin biopsy at the DC injection site was performed in all patients three days after the third vaccination.

Magnetic Resonance Imaging (MRI) and response evaluation

Patients underwent conventional contrast-enhanced MRI within two days after surgery, two days before the first vaccination, every two months, or in case of clinical indication. Tumor volumes were determined on the 3D post gadolinium T1 weighted images by manually outlining the enhancing portion of the lesion in MRIcro (http://www.mricro.com) as previously described[9]. To calculate the total enhancing volume of the tumor, the number of enhancing voxels

was multiplied by the voxel size. Disease progression was defined according to RANO criteria.

Immune monitoring

Immune monitoring was performed on the whole blood of each patient before, during and after DC vaccinations. Briefly, 100 μL of whole blood was incubated with 10 μL of conjugated primary antibodies for 10 min at 4°C in the dark. The lysis of erythrocytes and the fixation of stained leukocytes were performed using the Uti-Lyse Erythrocyte Lysing Reagent (Dako) according to the manufacturer's instructions for the "no wash" staining procedure. CD3, CD4, CD8 CD56 and CD45 mAbs (Miltenyi Biotec) were used to identify the T and NK cells. Peripheral blood lymphocytes (PBLs) from each patient were frozen before, after each vaccination and at the follow-up if performed and at the time of the biopsy for V-DENDR2 patients. The memory status of CD8+ and CD4+ T cells was evaluated using anti-CD3, CD8, CD4, CD45RA and CCR7 mAbs (Miltenyi Biotec). Non-viable cells were discriminated by using the Viobility dye (Miltenyi Biotec). The effector and central memory were also investigated using anti-KLRG1, CD38, CD127, CD25 mAb (Miltenyi Biotec). The IFNγ expression was assessed on thawed PBLs after restimulation with 50ng/ml phorbol myristate acetate (PMA), 1μg/ml Ionomycin and 10μg/ml Brefeldin A for a total of 4 hours. Lymphocytes were then fixed and permeabilized using the BD Cytofix/Cytoperm solution kit (BD Biosciences) and intracellular stained with IFNγ mAb (Miltenyi Biotec) according to the manufacturer's instructions. Acquisition of stained samples was performed using a MACSQuant (Miltenyi

Biotec) flow cytometer, and data were analyzed using the Flowlogic software (version 7.2, Miltenyi Biotec).

Immunohistochemistry

Cryostat frozen skin biopsies were sliced into 10μm-thick sections and fixed in acetone or 10% neutral buffered formalin. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water. Slides were treated with 1% bovine serum albumin (BSA) (Santa Cruz Biotechnology) and 5% normal goat serum in PBS containing 0.05% Triton X-100 (Sigma-Aldrich) and incubated in a closed humid chamber for 30min at room temperature. Sections were incubated with anti-CD4 (1:10, Dako) and anti-CCL3 (1:20, Thermo Fisher) antibodies overnight at 4°C. Staining was detected using the EnVision + System-HRP–labeled polymer secondary antibodies for 1 hour at room temperature and then the chromogen DAB/substrate reagent (Dako). Slides were counterstained with hematoxylin (Sigma-Aldrich), dehydrated, and mounted. After counterstaining with hematoxylin, sections were examined using a Leica microscope and analyses performed on 2 independent fields per section.

Real time-PCR

Total RNA was extracted from PBLs using Trizol reagent (Life Technologies). Total RNA was reverse-transcribed using a High Capacity cDNA Synthesis KIT (Applied Biosystems-Life Technologies). The expression of EOMES, ID3, TBET, PRDM1 genes was detected by SYBR Green chemistry performed on ViiA7 Real-Time PCR system (Life Technologies), and normalized relative

to β-actin. The RNA from the first vaccine was used as the calibrator for the calculation of fold expression levels with the ΔΔCt method.

Oligo Sequences:

EOMES	FW: ATGGGTGACCTGTGGCAAAG
	RV: TCCTGTCTCATCCAGTGGGA
ID3	FW: CAGCGCGTCATCGACTACAT
	RV: TGACAAGTTCCGGAGTGAGC
TBET	FW: CAAAGGATTCCGGGAGAACT
	RV: TAGTGATCTCCCCCAAGGAA
PRDM1	FW: GTGTGGTATTGTCGGGACTTTG
	RV: CAGTGCTCGGTTGCTTTAGAC

Statistical analysis

The Wilcoxon signed rank test was used to test the significance of differences between markers at various time points. All p values were two-sided. The chi-square or Fisher exact tests were used to examine the differences in categorical variables between groups. For efficacy evaluation, only patients that underwent at least to three vaccination doses are considered. Overall Survival (OS) 9 months, from surgery for disease recurrence to death due to any cause or last follow-up (censored), was considered as a relevant endpoint. In both studies, patients surviving more or less than 9 months were defined to distinguish long-term survivors (OS>9) and short-term survivors (OS≤9), respectively. Kaplan-Meier method was used to estimate OS. The log rank test assessed differences in survival. All statistical analyses were performed using Prism 5.03 software.

Figures

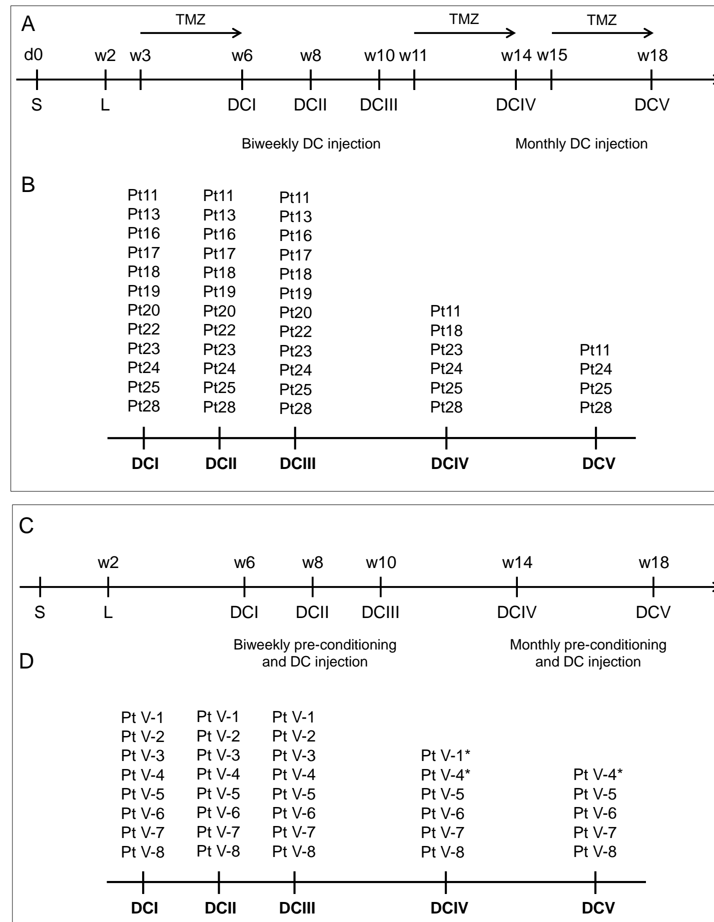
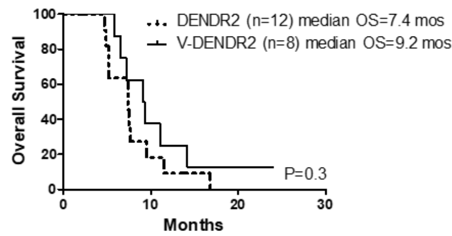


Figure 1 Treatment protocol for DENDR2 patients (A, B) and for V-DENDR2 patients (C, D) showing the timing before, during and after the DC administrations. S= surgery; L= leukapheresis; DC= dendritic cells; TMZ= temozolomide, w=week.

Patient	Age/ Gender	KPS	n. of TMZ cycles after 2nd surgery	n. of vaccinations (tot n=5)	MGMT (Met≥0.1)	Immune response[§]	OS (mts)
11	66/M	90	2	5/5	M (8.370)	No	9.5
13	61/F	70	2	3/5	NA	No	7.4
16	57/F	60	2	3/5	U (0.095)	No	4.7
17	61/M	60	1	3/5	M (12.894)	No	4.9
18	54/M	90	3	4/5	U*	No	7.7
19	58/M	90	1	3/5	U (0.017)	No	11.5
20	68/F	70	2	3/5	U (0.066)	No	5.2
22	45/F	80	2	3/5	M (0.730)	No	5.2
23	60/M	70	3	4/5	U (0.060)	No	7.4
24	51/M	80	3	5/5	U (0.000)	No	7.5
25	42/M	80	3	5/5	M (0.390)	No	16.8
28	54/F	80	3	5/5	U (0.020)	No	9.3
V-1	44/M	70	/	4/4	M (0.320)	Yes	9.3
V-2	56/M	100	/	3/5	M (1.560)	No	5.8
V-3	39/M	90	/	3/5	U (0.010)	No	7.2
V-4	56/M	90	/	5/5	M (0.130)	Yes	11.1
V-5	61/M	70	/	5/5	U (0.000)	No	6.5
V-6	34/F	100	/	5/5	U(0.050)	Yes	>24.0
V-7	69/F	80	/	5/5	M (2.120)	Yes	9.1
V-8	44/M	100	/	5/5	U (0.000)	Yes	14.2

Table 1 Abbreviations: KPS: karnofsky performance score, TMZ: temozolomide, RT: radiotherapy, MGMT: O (6)-Methylguanine-DNA Methyltransferase, OS: overall survival.

[§] Significant activation of T cell response; * Immunohistochemistry analysis.



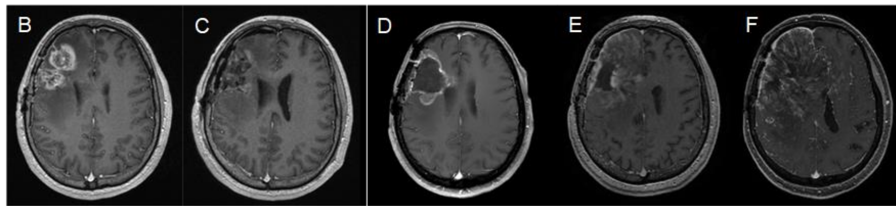


Figure 2 Survival analysis and MRI. (A) Kaplan Meier curves show the OS of DENDR2 patients compared to the OS of V-DENDR2 patients. (B-F) Patient V-8. Contrast-enhanced T1 w.i. MRI (small box, pre-contrast T1 w.i.); (B) before surgery for recurrence: Dec 28, 2016; (C) after surgery for recurrence: Dec 30, 2016; (D) at the time of first DC vaccination: Feb 21, 2017; (E) two months after the fifth and last DC vaccination: Jul 17, 2017; (F) disease progression: Dec 12, 2017.

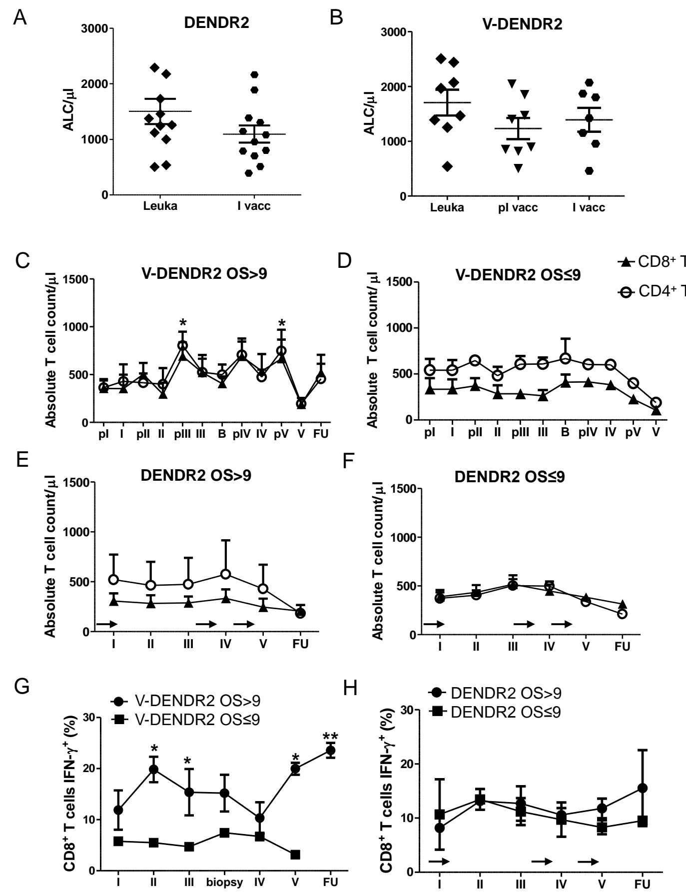


Figure 3 Absolute T cell counts before and after treatment. (A, B) Absolute lymphocyte counts (ALCs) in the peripheral blood of patients at the time of the leukapheresis (leuka) and at the time of the first vaccination (I vacc), after TMZ treatment, in DENDR2 patients (A); at leuka, before TT pre-conditioning and first vaccination (pI vacc) and at the time of the first vaccination (I vacc) in V-DENDR2 patients (B); (C-F) Time course of $CD8^+$ and $CD4^+$ absolute counts of V-DENDR2 OS>9 (C, n = 5) and OS≤9 (D, n = 3) patients over the treatment, including the time of the skin biopsy (B), (* P<0.01 vs. pre-I vaccine, at the time of TT pre-conditioning), and of

DENDR2 OS>9 (E, n=4) and OS≤9 (F, n=8) patients over the treatment. Data are presented as mean ± SEM; (G, H) Kinetics of the frequency of $CD8^+$ T cells expressing IFNγ assessed by flow cytometry of V-DENDR2 (G) and DENDR2 patients (H) (*P<0.01, **P<0.005, vs. I vaccination). The arrows indicate the TMZ administrations. Data are presented as mean ± SEM. Representative dot plots are shown in **Fig. S1**.

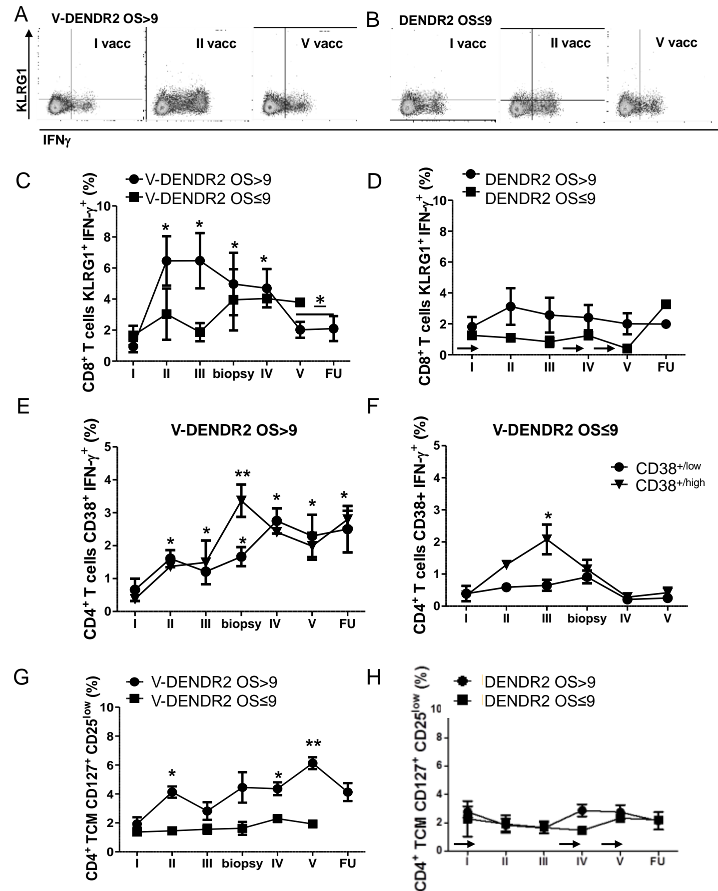


Figure 4 Identification of effector to memory transition and T helper memory response. (A, B) Exemplificative dot plot representing the double-positive KLRG1/IFNγ cells gated in CD45/CD3/CD8^{+} T cells in a V-DENDR2 OS>9 (A, Pt V-6) and in a DENDR2 OS>9 (B, Pt25); (C, D) Kinetics of the frequency of CD8^{+} T cells expressing KLRG1 and IFNγ in V-DENDR2 (C), and DENDR2 patients (D) over the treatment (including the time of biopsy in V-DENDR2 patients and follow up (FU) when performed) (*P<0.01 vs I vaccination, underlined asterisk vs. II vaccination); (E-F) Kinetics of the frequency of CD38$^{+/high}$ and CD38$^{+/low}$ expressing IFNγ evaluated in

$CD45/CD3/CD4^+$ Tcm and Tem subset, after in vitro re-stimulation, in V-DENDR2 OS>9 (E, *P<0.01 , **P<0.005 vs I vaccination) and OS≤9 (F); (G, H) Time course of $CD4^+$ $CD127^+$ $CD25^{low}$ T cells analysis in V-DENDR2 (G, *P<0.01 , **P<0.005 vs I vaccination) and DENDR2 patients (H). The arrows indicate the TMZ administrations.

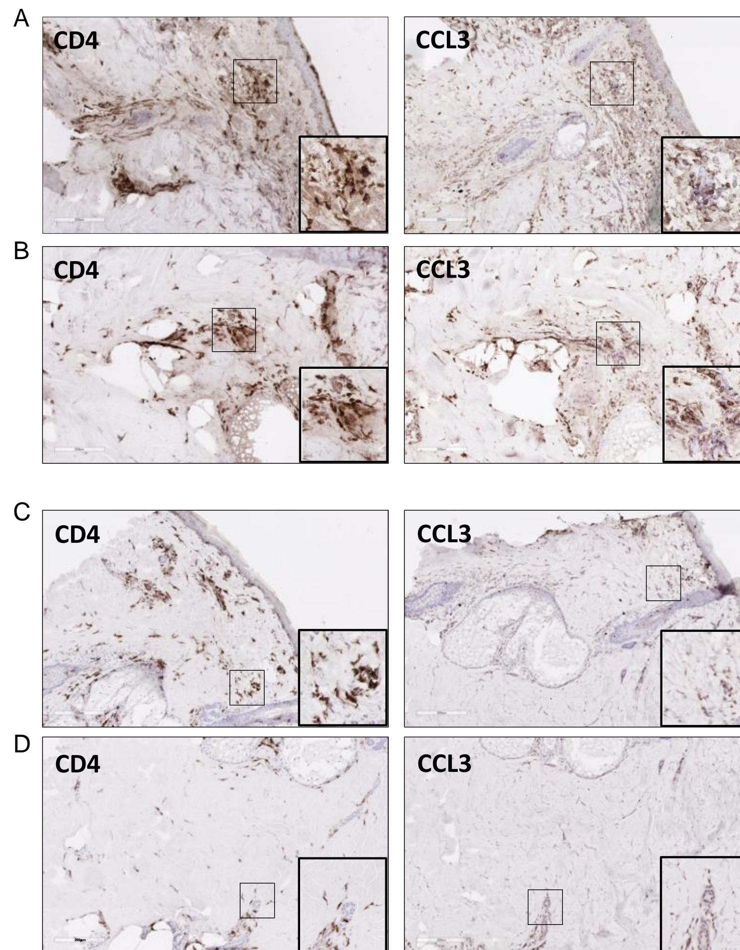


Figure 5 Investigation of the CCL3-expressing CD4$^+$ T cells in skin biopsies. Two V-DENDR2 and two control patients have been investigated, and representative images are displayed. (A, B) Rectangles indicating the same areas in adjacent sections of the skin biopsies show a robust dermal infiltration of CD4$^+$ T cells (left panels) expressing CCL3 (right panels). (C, D) In the control skin biopsies, the IHC reveal a moderate (C) and low (D) infiltration of CD4$^+$ T cells and negative for CCL3.

Supplementary material

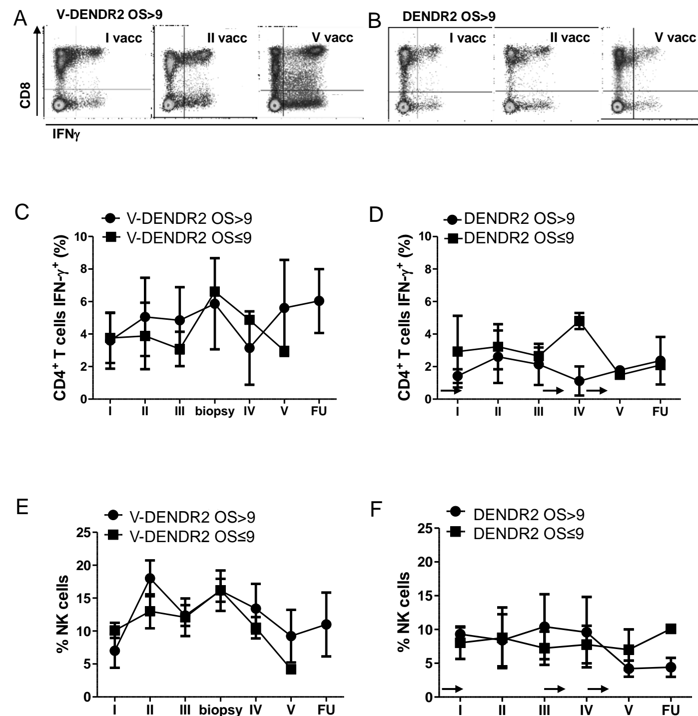


Figure S1 Characterization of effector response. (A, B) Representative dot plots showing double positive CD8/IFNγ T cells gated on CD45/CD3 at first, second and fifth vaccine assessed by flow cytometry in V- DENDR2 patients. (C, D) Kinetics of frequency of $CD4^+$ T cells expressing IFNγ assessed by flow cytometry in V-DENDR2 (C) and DENDR2 patients (D) over the treatment (including the time of biopsy in V-DENDR2 patients and FU when performed). (E, F) Kinetics of frequency of NK cells evaluated by flow cytometry in V-DENDR2 (E) and DENDR2 (F) patients over the treatment (including the time of biopsy in V-DENDR2 patients and FU when performed).

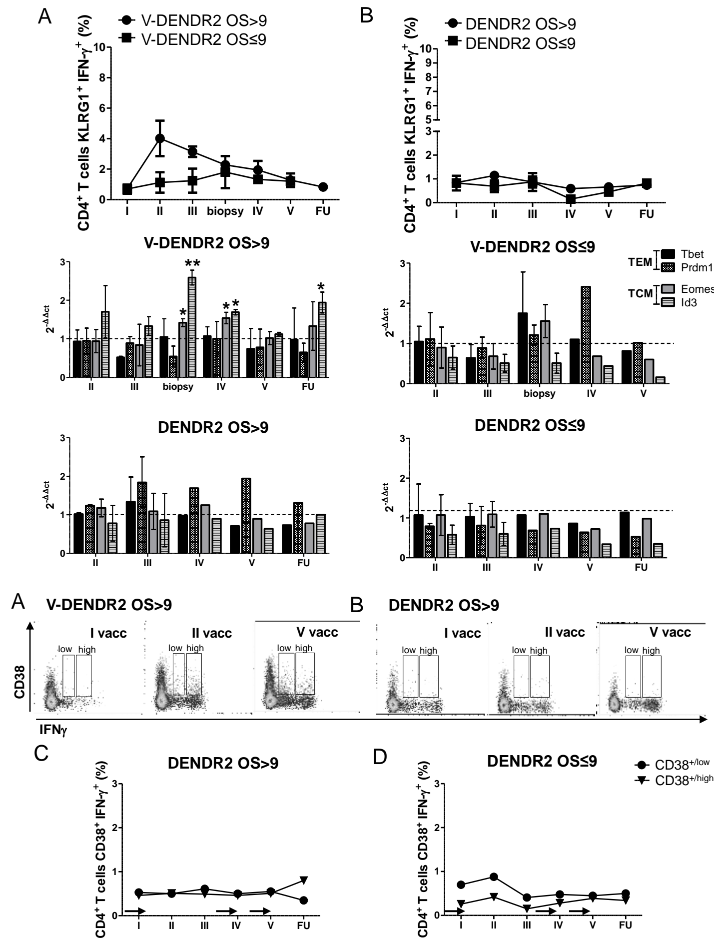


Figure S2 Characterization of effector to memory transition and T helper memory response. (A, B) Kinetics of frequency of CD4^{+} T cells expressing KLRG1 and IFNγ assessed by flow cytometry in V – DENDR2 (A) and DENDR2 (B) patients over the treatment (including the time of biopsy in V-DENDR2 patients and FU when performed). (C, D) Bar graphs showing the relative expression of T central memory (EOMES, ID3) or T effector memory (TBET, PRDM1) associated genes in PBLs over the treatment in V- DENDR2 (C) and DENDR2 (D) patients, evaluated by real time PCR. The relative expression of genes was compared with that detected at the

first vaccine (dotted line) (* $P<0.01$ and ** $P<0.005$ vs first vaccine). (E) Representative dot plots showing double-positive CD38/IFNγ T cells gated on CD45/CD4 at first, second and fifth vaccine assessed by flow cytometry in V-DENDR2 patients. (F, G) Kinetics of frequency of $CD4^+$ T cells expressing $CD38^{high/low}$ and IFNγ assessed by flow cytometry in DENDR2 patients over the treatment (including the FU when performed).

Patient	Treatments after PD at the end of IT
11	Bevacizumab (2 cycles)
13	Bevacizumab (4 cycle)
16	No treatment
17	No treatment
18	FTM (3 cycles)
19	Bevacizumab (>10 cycles) + CCNU
20	CCNU (1 cycle)
22	CCNU
23	CCNU+ PCZ (2 cycles)
24	Bevacizumab (3 cycles)
25	TMZ 21/28, from DENDR2 and on (tot 4 cycle); ETP
28	PCV /RT
V-1	PCV (2 cycles)
V-2	PCV (2 cycles)
V-3	PCV (2 cycles)
V-4	No treatment
V-5	No treatment
V-6	No treatment
V-7	No treatment
V-8	No treatment

Table S1 Treatments after DC immunotherapy. Abbreviations. FTM: fotemustine; CCNU: Lomustine; TMZ: temozolomide; PCZ: procarbazine; ETP: etoposide; PCV: Procarbazine, CCNU, and Vincristine; RT: radiotherapy

Adverse Events (AE)	No. of events		Surgery-related	IT-related	TMZ-related
	D2	V-D2			
Brain edema	2	0	NO	YES	NO
Fever	1	0	YES	NO	NO
Nausea	1	2	YES	NO	NO
Vomiting	0	1	NO	YES	NO
Pain	2	0	YES	NO	NO
Asthenia	2	0	NO	YES	YES
Headache	1	3	YES	NO	NO
Seizure	1	3	NO	YES	NO
Neurosurgical wound complication	2	0	YES	NO	NO
Surgical site collection	2	0	YES	NO	NO
Dysphasia	0	2	NO	YES	NO
Dizziness	0	1	NO	YES	NO
Cognitive disturbance	0	2	NO	YES	NO
Hypostenia	0	3	NO	YES	NO
Intracranial bleeding	0	1	NO	NO	NO
Pulmonary embolism	0	1	NO	NO	NO
Pneumonia	0	1	NO	NO	NO
HEAMATOLOGICAL TOXICITIES:					
Thrombocytopenia	2	0	NO	NO	YES
Lymphopenia	10	2	NO	NO	YES
Anemia	5	2	YES	NO	YES
Leucopenia	1	0	NO	NO	YES
Neutropenia	1	0	NO	NO	YES
Liver abnormality	1	0	NO	NO	YES
VACCINATION-SITE REACTIONS					
Eythema	0	6	NO	YES	NO
Pruritus	0	0	NO	YES	NO
Pain and induration	0	5	NO	YES	NO

Table S2 Adverse events. Abbreviations. IT: immunotherapy; TMZ: temozolomide; No.: numbers

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Chapter 4

1. Summary

Cancer immunotherapy has made a significant impact on the treatment of cancer patients in the last years. However, despite its clear efficacy in several malignancies, only a fraction of patients benefit from this approach. This condition has generated considerable interest in combinatorial strategies to improve immunotherapeutic approaches. The idea that some chemotherapeutic agents can influence the immune response either by inducing the immunogenic death of tumor cells or by modulating key cells for immune suppression or activation[1], has paved the way to a potential synergy between chemotherapy and immunotherapy.

In two clinical studies, that have been active in our Institute, dendritic cell (DC) – vaccinations were combined with temozolomide (TMZ), the standard chemotherapeutic treatment for glioblastoma (GBM) patients, administered as an adjuvant.

In DENDR1 clinical study (EUDRACT No 2008-005035-15), patients with first diagnosis of GBM treated with dendritic DC immunotherapy combined with TMZ showed a specific and long- lasting NK cell response, that positively impacts on survival. However, we observed a clinical efficacy in 45% of the treated patients. In particular, we observed a failure of $CD8^+$ T cell response impaired by TMZ administration. Moreover, TMZ action interfered by preventing the generation of a $CD8^+$ T cell memory status. According to our preclinical data[2], we found that resistance to TMZ was associated with the expression of a multidrug resistance protein ABCC3 in NK but not $CD8^+$ T cells[3].

In DENDR2 clinical study (EUDRACT No 2008-005038-62), patients with recurrent GBM were treated with DC immunotherapy in combination with dose-dense TMZ. The trial is now closed due to the failure of activation of a specific immune response and survival benefit.

From these observations, and given that recurrent GBM does not have a standard treatment and is associated with a poor prognosis, we moved to delineate a new combinatorial strategy. Recent results indicated that pre-conditioning the vaccine site with tetanus/diphtheria (Td) toxoid antigen may represent a strategy to improve cancer immunotherapy in GBM patients[4].

In the variant (V) – DENDR2 pilot study, the vaccine site was pre-conditioned with the recall antigen tetanus toxoid (TT) 24 hours before each DCs administration. TMZ was avoided according to the DENDR1 results. We analyzed the effector response and we found a significant $CD8^+$ T cell activation, due to TMZ absence, and an effector to memory transition based on the loss of KLRG1 expression. Moreover, we observed an increase of vaccine-specific $CD4^+$ T cells accompanied by an expansion of TT-specific $CD4^+$ T cells, based on CD38 and CD127 expression. Interestingly, $CD4^+$ T cells expressing the CCL3 chemokine infiltrated the injection site, supporting a potential increase in DC migration. Overall, this immunological status had a better impact on survival, considering that 62.5% of patients reached the endpoint OS9.

These findings highlighted the importance to delineate potential combinatorial strategy to improve immunotherapy for GBM. Particularly, metronomic schedules of TMZ (and possibly

cyclophosphamide) may ameliorate and complete the immune response with the contribution of $CD8^+$ T cells[5–7]. On the other hand, giving the fact that the methylation of the DNA repair gene MGMT is a favorable prognostic marker and predictive of higher response to TMZ[8], this subgroup of patients will benefit from standard, higher dosage TMZ treatment. Moreover, the vaccine site pre-conditioning seems to be a promising approach to stimulate a long-lasting anti-tumor immune response, even in patients with recurrent GBM. In this scenario we plan to expand the V-DENDR2 experience, designing a larger study to better characterize the anti-tumor immune response, especially focusing on the $CD4^+$ T helper cell population, that sustain the $CD8^+$ T cell response.

Altogether these results may open the way to further studies in order to increase the DC response and to other immunotherapy approaches by increasing neoantigen presentation by local chemotherapy or even radiotherapy.

2. Conclusions

Cancer treatments, engaging the immune system to fight against tumors, have demonstrated to be effective against several malignancies, especially melanomas[9]. That is not the case of glioblastoma (GBM), the most common and aggressive brain cancer in adults. Despite continued efforts to develop new therapies, none has significantly improved patients survival, which is less than 2 years.

Dendritic cells (DCs), powerful antigen-presenting cells, are an important tool for cancer immunotherapy. DC immunotherapy has been proven effective in prolonging survival of GBM patients[10], but the tumor volume at the time of vaccine and the potential anergy and low frequency of tumor-specific T cells could limit its efficacy. This condition has generated a great interest in the designing of combinatorial strategies that is not a simple endeavor or addition of different agents. The tumor microenvironment is a very complex system and the delineation of the optimal combination should take into account this complexity. Chemotherapy has been proposed as an adjuvant able to influence the immune response, by inducing immunogenic death of tumor cells or by modulating key cells for immune suppression or activation[11]. Temozolomide (TMZ) exerts its effect through direct killing of tumor cells, but several data indicate that it can also influence the immune cells by inducing lymphopenia and impacting on tumor microenvironment. However, clinical data on the combination of TMZ with immunotherapy are limited.

In the past ten years, two clinical studies have been activated (DENDR1 EUDRACT No 2008-005035-15; DENDR2 EUDRACT

No 2008-005038-62), in which DC immunotherapy is administrated with TMZ as an adjuvant. The immune-monitoring and the characterization of effector populations is an important tool to understand the immune response induced by the vaccines. We expected that, upon intradermal injection, DCs migrated to lymph nodes and presented antigen to $CD4^+$ and $CD8^+$ T cells. This interaction is essential to activate clonal expansion of cytotoxic effector cells with the induction of effective and durable immune responses. Our data indicated that this strategy is able to activate an anti-tumor immune response, mediated mainly by NK cells increase in peripheral blood after DC vaccination in DENDR1 patients. We demonstrated that NK cells are critical immune effectors during DC vaccination: in responder patients, they significantly expanded after the second vaccination and durably expressed IFNγ in response to and until the end of the treatment. Interestingly, we have some evidence that the cytotoxic NK cell subset $CD56^{dim}$ $CD16^+$ increased significantly in responder patients during the treatment (data not shown), supporting again the primary role of NK cells in mediating the anti-tumor immune response, which is strongly related to better survival. These results supported our published data regarding recurrent GBM treated with DC immunotherapy[12]. Previous work showed that NK cells contribute to DC immunotherapy enhancing the ability of DCs to produce cytokines and promoting effective T cell-based antitumor responses[13]. Nevertheless, preclinical data suggested that NK cells may compromise the $CD8^+$ T cell priming through PD1- PDL1 interactions with DCs[14]. This is what probably happened in our patients.

Another important aspect to consider is the TMZ action on the immune system. We found that TMZ administration negatively impacted on $CD8^+$ T cell activation and consequently on memory formation, that is a critical requirement for an efficient and long-lasting anti-tumor immune response. Moreover, our data indicated that the multidrug resistance protein ABCC3 is up-regulated in NK but not in $CD8^+$ T cells during TMZ treatment, according to our preclinical data[2]. Overall, this evidence suggested that the schedule of TMZ should be re-evaluated in order to complete the anti-tumor immune response with the contribution of $CD8^+$ T cells.

In the DENDR2 clinical study, we did not observe the same response. Patients with recurrent GBM failed to activate a significant anti-tumor immune response, which led to an advantage in terms of survival. Interestingly, we observed not only a total absence of $CD8^+$ T cell response but also a lack of NK cell activation. This was in contrast with the data emerged from the DENDR1 study. Probably, NK cells became exhausted upon extended stimulation and exposure to TMZ. Several data indicate that tumor progression usually leads to NK cell exhaustion, limiting their anti-tumor potential[15]. However, patient 28 exhibited an NK cell activation during the treatment, immediately after the second vaccination lasting over time, after the end of the treatment. This is the only patient treated with the topoisomerase II inhibitor etoposide after the vaccination schedule. A number of studies indicate that etoposide induces a DNA damage that in turn lead to NKG2D ligands upregulation which is important for NK cell recognition and killing of tumor cells[16,17]. Altogether these findings might support the greatest survival observed among DENDR2 patients

(OS=16.8 mos), further strengthening the importance and the positive impact of NK cells on anti-tumor immune response, even in absence of methylation of the MGMT promoter, which is predictive of higher response to TMZ[8].

Take into consideration that currently there is not a standard of care for recurrent GBM and that in DENDR1 patients TMZ negatively impacts on $CD8^+$ T cell-mediated immune response, we looked for other strategies able to improve the anti-tumor immune response. A previous work underlined the importance of vaccine site pre-conditioning to enhance the efficacy of DC immunotherapy[4]. Our pilot study for patients with recurrent GBM, named variant (V) – DENDR2, provided a vaccine site pre-conditioning with the tetanus toxoid (TT) in combination with DC immunotherapy and in absence of TMZ. Data showed an encouraging gain in patients survival, together with a strong $CD8^+$ T cell response and memory formation. It is well known that TT can attract TT-specific $CD4^+$ T cell at the site of vaccination, promoting CCL3 chemokine release, that upregulates the expression of CCL21. In this scenario, DCs migrate into the lymph node where they interact with vaccine-specific $CD8^+$ and $CD4^+$ T cells. Our preliminary data obtained on the skin biopsies of V-DENDR2 patients showed the presence of $CD4^+$ T cells expressing CCL3. However, further staining will be performed including $CD8^+$ T cells or macrophages markers vs. CCL3 to confirm the specificity of $CD4^+$CCL3 co-expression at the injection site in response to TT.

Moreover, our analysis focused on $CD4^+$ T cell subset, revealed a vaccine-specific $CD4^+$ T cell activation paralleled by an increase in bystander $CD4^+$ memory T cell response during the treatment,

supporting the anti-tumor $CD8^+$ T cell response[18]. Indeed, as previously demonstrated, the recall response can induce proliferation of previously activated $CD4^+$ T cells specific for unrelated antigen[19]. Most important, the higher frequency of $CD4^+$ T cells reflected the clinical advantage in patients that reached OS9.
These findings suggest that pre-conditioning of the vaccine site could be a good tool to improve the efficacy of DC immunotherapy, contributing to effector response and memory status generation.

The survival advantage observed in both studies took into account the published data from the Brain Cancer Register of our Institution, in which available information was compared to assess the predictive effect of first-line treatments on OS and PFS and the effects of reoperation and second-line treatments on survival after recurrence. Median OS in patients with GBM recurrence was 8.9 months (95% CI 8.0-9.8 mos); however IDH1-2 mutations, a powerful positive prognostic factor, were not investigated in these patients[20].

A growing body of evidence indicates that, in several tumors, a high number of mutations significantly improve the efficacy of cancer immunotherapy with checkpoint inhibitors[21,22]. Particularly, a high mutational load seems to be linked to a high number of neoantigens. The advent of next-generation sequencing technologies have made possible to easily identify this source of antigens[23], that can be exploited for personalized neoantigen-based immunotherapies. To date, the heterogeneity of tumor mutational load has limited the application of mutanome - direct therapy in tumors with a low mutational load[24]. However, recent data from Zacharakis and

colleagues have subverted this view and highlighted the potential of a treatment based on neoepitope- reactive tumor infiltrating lymphocytes (TILs), for tumors with a low mutational load[25].

Accumulating data indicate that DC immunotherapy may show evidence of increased efficacy in presence of hypermutations. Specifically, patients with advanced melanoma, treated with DCs, displayed an augment in naturally occurring neoantigen-specific immunity and revealed (HLA) class I restricted neoantigens[26]. Other studies evidenced an increase of TILs abundance induced by DC immunotherapy, in such cold tumors as gliomas[27]. Interestingly, recent data from Tanyi JL and colleagues demonstrated that DC vaccination was able to enhance the T cell response against mutated neoepitopes, including the priming of novel high-avidity T cell clones[28].

Taken together these observations encourage the pursuit of DC vaccination for GBM, taking into account the importance of hypermutations and consequently, neoantigen- specific T cell response, in a context of personalized medicine.

3. Future perspectives

Immunotherapy represents one of the most important advances in cancer treatment in the past decades. Such an approach has deeply revolutionized the treatment of certain types of cancer. In 2013, the Science magazine named cancer immunotherapy the *"Breakthrough of the Year"*, underlining the power of this approach to treat cancer. Five years later, James P. Allison and Tasuku Honjo have been awarded the Nobel Prize for Medicine, for their work on unleashing the body's immune system to attack cancer.
Despite this enormous power, immunotherapy approach is currently effective only for a fraction of patients, thus the use of different therapeutic strategies simultaneously is expected to advance rapidly.

Several data supported the immune-stimulatory potential and the ability to favor the immune response against tumor of chemotherapy[11]. Our data indicated that adjuvant TMZ limited the anti-tumor immune response stimulated by DC immunotherapy, impairing T cell response in primary glioblastoma (GBM). In this scenario, the schedule of TMZ administration should be re-evaluated, in order to favor the cytotoxic action of $CD8^+$ T cells against the tumor. Methylation of the DNA repair gene MGMT is a favorable prognostic marker and is also predictive of higher response to TMZ[8]. We found hypermethylation of the MGMT promoter in 25% of treated patients and it was associated with longer PFS and OS. Thus, in order to delineate a new schedule, we will consider administering TMZ to methylated patients only, who may take advantage in terms of survival.

Furthermore, hypermutations may increase the number of neoantigens: a high mutational load makes neoantigens visible to the immune system, thus eliciting a strong T cell response.
GBM with functional MGMT are resistant to TMZ treatment. On the other hand, in presence of MGMT methylation, TMZ resistance may induce hypermutation in gliomas[29], leading to neoantigen generation that can be recognized by $CD8^+$ T cells[24]. Recent data from Wang et al. unveiled a correlation between hypermutation and $CD8^+$ T cell infiltration. Specifically, in five GBM patients the frequency of $CD8^+$ T cells was significantly higher at recurrence in comparison to their primary tumors[30]. Therefore, these observations suggest that patients with hypermutated tumors may take more advantage from $CD8^+$ T cell antitumor response. Our data demonstrated that TMZ might significantly impair the immune response induced by DC immunotherapy. Based on this evidence, the absence of TMZ might favor the recognition of neoantigens by T cells stimulated by DC immunotherapy[28].

Finally, we still do not have a standard treatment for recurrent GBM. Data from the pilot study variant (V) – DENDR2 suggested that pre-conditioning of the injection site with tetanus toxoid (TT) showed increased survival and signs of immune responses. This evidence encourages us to deeply investigate the contribution of TT to enhance DC immunotherapy effectiveness, designing a clinical study for patients with recurrent GBM in the next future. Moreover, our data supported the view that this treatment was able to prime and boost an immune response against tumor-specific antigens. T lymphocyte clones specific for these antigens might then invade the tumor and

mediate its destruction. In order to evaluate the specificity of $CD8^+$ T cells activated by the vaccination, it might also be useful to perform a TCR sequencing in long-term survivors as previously shown in GBM and other tumors treated with immunotherapy[27,31].

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5. Publications

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2. Pellegatta S, Savoldo B, Di Ianni N, **Corbetta C**, Chen Y, Pollo B, Ferrone S, DiMeco F, Finocchiaro G, Dotti G. *Constitutive and TNFα-inducible Expression of Chondroitin Sulfate Proteoglycan 4 in Glioblastoma and neurospheres: Implications for CAR-T Cell Therapy*. Science Translational Medicine. 2018 Feb 28; 10(430). pii: eaao2731. doi: 10.1126/scitranslmed.aao2731.

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