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**Review**

## **Recent advances and future of immunotherapy for glioblastoma**

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**Key words:** Glioma, immunotherapy, gene therapy, cancer vaccines, passive immunotherapy, checkpoint blockade

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## **Abstract**

**Introduction:** Outcome for glioma (GBM) remains dismal despite advances in therapeutic interventions including chemotherapy, radiotherapy and surgical resection. The overall survival benefit observed with immunotherapies in cancers such as melanoma and prostate cancer has fuelled research into evaluating immunotherapies for GBM.

**Areas covered:** Preclinical studies have brought a wealth of information for improving the prognosis of GBM and multiple clinical studies are evaluating a wide array of immunotherapies for GBM patients. This review highlights advances in the development of immunotherapeutic approaches. We discuss the strategies and outcomes of active and passive immunotherapies for GBM including vaccination strategies, gene therapy, check point blockade and adoptive T cell therapies. We also focus on immunoediting and tumor neoantigens that can impact the efficacy of immunotherapies.

**Expert opinion:** Encouraging results have been observed with immunotherapeutic strategies; some clinical trials are reaching phase III. Significant progress has been made in unraveling the molecular and genetic heterogeneity of GBM and its implications to disease prognosis. There is now consensus related to the critical need to incorporate tumor heterogeneity into the design of

therapeutic approaches. Recent data also indicates that an efficacious treatment strategy will need to be combinatorial and personalized to the tumor genetic signature.

**Keywords:** Glioma, Immunotherapy, Cancer Vaccines, Checkpoint blockade, Gene Therapy,

## 1. Introduction

Gliomas are the most frequently diagnosed human primary brain tumors<sup>1</sup>. Glioma is derived from the greek word for “glue”, as glial tumors are derived from CNS cells that form the scaffolding and structural support for neurons. Gliomas are diagnosed according to the predominant cell population (astrocyte or oligodendrocyte) and are graded according to pathologic features that correlate with tumor aggressiveness and clinical outcome, including nuclear atypia, microvascular proliferation, hemorrhages and necrosis<sup>1</sup>. Lower-grade gliomas (WHO grade II/III) are molecularly similar and frequently progress to glioblastoma (GBM, WHO grade IV).

Recently, efforts have been made to sub-group grade II-III (“lower grade”) gliomas based on shared molecular features. In particular, sub-groups are based on the molecular status of: (1) deletion of chromosomes arms 1p and 19q (1p/19q co-deletion), (2) mutation in codon 132 of the isocitrate dehydrogenase 1 gene (*IDH1*), and (3) activating mutations in the promoter of the *TERT* gene, which encodes telomerase<sup>2, 3</sup>. Sub-grouping according to these features is predictive of histologic sub-type and prognosis, and molecular characteristics are increasingly used in the diagnostic work-up of gliomas (**Table 1**)<sup>4</sup>. For example, *IDH1* mutational status has become a key feature in determining the prognostic and biologic features of lower-grade glioma<sup>2, 3, 5, 6</sup>.

*IDH1* mutation is found in a majority of lower-grade glioma and secondary GBM, and is a positive prognostic variable<sup>2, 3</sup>. Gliomas which carry 1p/19q co-deletion are oligodendrocytic in lineage, and carry the best prognosis and response to alkylator-based chemotherapy and radiation<sup>7</sup>. Lower-grade *IDH1*-mutated gliomas without co-deletion are predominantly astrocytic tumors, and frequently carry concurrent mutations in the tumor suppressor *TP53* and in the histone chaperone protein *ATRX*<sup>4, 5</sup>. Mutation (and loss) of *ATRX* was recently described to promote both glioma tumor growth and genetic instability<sup>8</sup>. The group of *IDH* mutated, not co-deleted lower-grade gliomas carry a moderate prognosis, while non-*IDH1*-mutated gliomas carry the worst prognosis<sup>2, 3</sup>. These molecular features are less prognostic in primary GBM, which carries a uniformly dismal prognosis<sup>2, 3</sup>.

Despite progress in understanding the molecular attributes of glioma, current treatments are sub-optimal and human glioma results in significant morbidity and mortality<sup>9</sup>. Prognosis in gliomas is correlated with the degree of maximal safe resection<sup>10, 11</sup>. However, complete resection of gliomas is rarely achieved due to tumor infiltration into normal tissue and/or proximity to critical motor/sensory tracts<sup>12</sup>. Radiation is effective in prolonging survival in GBM and may be beneficial in some grade II/III gliomas<sup>13</sup>. Gliomas generally invade and grow locally; therefore radiation is given focally to involved areas.

Chemotherapy is only minimally effective in the treatment of gliomas. The addition of the alkylating agent temozolomide (TMZ), during and after radiation, prolongs survival in GBM, especially those with promoter methylation of the DNA-damage repair protein O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT)<sup>9</sup>. Other cytotoxic agents and regimens have displayed efficacy in certain lower-grade gliomas<sup>14</sup>, but the efficacy of most chemotherapeutic agents is hindered by the difficulty of delivering agents to tumor cells in the brain parenchyma<sup>15</sup>.

Thus there is an urgent need for the development of efficacious multi-pronged therapies that are tailored to the unique facets of GBM biology.

Recent clinical data has caused a strong interest in the development and evaluation of immunotherapeutic approaches for GBM<sup>16-18</sup>. Approval of sipuleucel-T (Provenge) for metastatic hormone resistant prostate cancer and ipilimumab (Yervoy) for metastatic melanoma by the FDA has validated the efficacy of immunotherapies in other cancers<sup>19</sup>. In addition, a growing body of evidence has demonstrated the prognostic impact of immune cell infiltrates in the tumor<sup>20, 21</sup>.

The CNS has been traditionally considered an immune privileged system. However, a growing body of evidence has challenged this concept lately<sup>18, 22-25</sup>. It has been shown that immune cells can cross the blood brain barrier to gain access to the brain parenchyma and can leave the CNS to reach the cervical lymph nodes. Also, the ventricles, meninges, and perivascular spaces lack blood brain barrier (BBB) and their immune-reactivity is not different from that in the periphery<sup>22-25</sup>. Considering that the immune system has access to the brain and that GBM expresses multiple tumor antigens that can be targeted by immunotherapeutic approaches, the development of these therapies has gained considerable interest over the last decade. Immunotherapeutic approaches aim to induce an adaptive immune response that specifically targets and kills GBM cells without affecting normal cells within the brain parenchyma. Although these strategies trigger antitumor immunity<sup>26</sup>, the clinical benefit has been lower than hitherto anticipated. The ability of the immunotherapeutic approach to inhibit the immunosuppressive tumor microenvironment may be crucial to yield tumor regression<sup>27, 28</sup>.

### **1.1 Mechanisms of immune suppression in GBM**

GBM cells actively contribute to the generation and maintenance of the immunosuppressive microenvironment<sup>29, 30</sup> (**Fig. 1**). These cells produce anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ , which inhibit effector T cell responses and APC function, while stimulating regulatory T cell (Treg) expansion and function<sup>31</sup>. Tregs are also recruited to the tumor microenvironment by the local production of chemokines, such as CCL2, by GBM cells<sup>32</sup>. Besides Tregs, myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) also infiltrate the GBM tumor microenvironment, inhibit anti-tumor immune responses, and support tumor progression<sup>33, 34</sup>. Modulation of the expression of membrane-bound molecules that interact with tumor infiltrating immune cells is another mechanism that inhibits the adaptive antitumor immune response against GBM. Differential expression of Human Leucocyte Antigen (HLA) molecules has been involved in the immunological escape of GBM. The expression of HLA class I is downregulated in GBM specimens when compared with lower grade astrocytomas, which has been involved in the evasion of cytotoxic T lymphocyte (CTL) responses<sup>35</sup>. Although downregulation of HLA class I reduces the immunogenicity of GBM cells, it could activate NK cell response against the tumor. However, GBM cells overexpress nonclassical HLA class I molecules, such as HLA-G and HLA-E<sup>36, 37</sup>, which are involved in the inhibition of NK and CTL responses to the allogeneic fetus during pregnancy. Changes in HLA class II expression have also been reported in GBM cells and have been associated with epigenetic changes in the class II transactivator (CIITA) promoter<sup>38</sup>.

GBM cells can also directly inhibit T cell function through the expression of negative regulators of T cell function. FasL-expressing GBM cells induce apoptosis of tumor-infiltrating T cells that express Fas<sup>39</sup>. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Programmed

death-1 (PD-1), Lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin mucin-3 (TIM-3) are negative regulators of the immune system and seem to have a crucial role in GBM immunological escape<sup>22</sup>. While CTLA-4 expression reduces the activation of naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>40-42</sup>, PD-1 and TIM-3 are markers of T cell exhaustion in activated lymphocytes<sup>43</sup>. LAG3 is a negative regulator of activated T and NK cell expansion during chronic inflammation and tumorigenesis<sup>44</sup>. These molecules not only inhibit effector T cell and NK cell activation and function, but they also promote Treg immunosuppressive function<sup>22</sup>. Considering that ligands of these proteins are overexpressed in GBM cells, i.e. PD-L1<sup>45</sup>, galectin-9<sup>46</sup>, they constitute valuable targets to overcome the immunosuppressive nature of GBM and improve the efficacy of immunotherapeutic strategies for GBM.

## **2. Immunotherapy approaches**

Immunotherapeutic strategies can be broadly divided into four major classes; (1) immunomodulatory strategies like the ones that target checkpoints using ipilimumab, (2) active immunotherapy such as with cancer vaccines and immune stimulatory gene therapy, (3) passive immunotherapies utilizing antibodies and (4) adoptive strategies such as the ones using chimeric antigen receptor (CAR) T cells.

### **2.1 Targeting immunosuppressive checkpoints**

T cells interact with Antigen-Presenting Cells (APCs) and tumor cells, providing signals to regulate immune response through stimulation or inhibition of T-cell activation<sup>47</sup>. These several ligand-receptor interactions are called checkpoint pathways. Checkpoint pathways are non-redundant and can be either co-stimulatory or co-inhibitory. While CD28, TNFRSF4 (OX40), CD40L, CD2 and CD137 enhance immune response, CTLA-4, PD-1, LAG-3, TIM-3 and TIGIT inactivate T lymphocytes<sup>48-53</sup>. Ligands for these immunosuppressive checkpoints are



often overexpressed in the GBM microenvironment to inhibit T-cell response against tumor cells, and can be targets for therapies.

CTLA-4 is expressed exclusively by T cells and inhibits their activation by dephosphorylating the CD3 $\zeta$  chain. CD28 and CTLA-4 share the ligands CD80 and CD86, but CTLA-4 binds them with higher affinity<sup>54, 55</sup>. Ipilimumab is a fully humanized monoclonal antibody against CTLA-4, approved in 2011 by the FDA and the European Medicines Agency for the treatment of metastatic melanoma which has shown benefits in patients with brain metastasis<sup>56-59</sup>. Extensive preclinical research has shown exciting results with CTLA-4 blockade<sup>19</sup>. Administration of CTLA-4 blocking antibodies improves the survival of animals bearing intracranial SMA-560 tumors<sup>60</sup>, anti-CTLA-4 antibodies in combination with IL-12 also led to the eradication of GL261 gliomas<sup>61</sup>, and the combination of CTLA-4 antibodies with vaccination with GMCSF expressing GBM cells was more effective than either treatment alone<sup>62</sup>. Ipilimumab has demonstrated encouraging results in patients with melanoma and brain metastases as a single agent therapy or in combination with radiotherapy<sup>18</sup>, and a clinical trial evaluating ipilimumab for recurrent and newly diagnosed GBM is currently recruiting patients (NCT02017717).

PD-1 binds to PD-L1 and PD-L2, expressed on surface of tumor cells<sup>50, 63</sup>, tumor-associated macrophages<sup>33</sup>, vascular endothelial cells<sup>64</sup> and some neurons of the tumor microenvironment<sup>65</sup>. Particularly, PD-L1 has been found to have increased expression in GBM patients<sup>45</sup>. In 2014, the FDA approved Pembrolizumab and Nivolumab for metastatic melanoma. Later in 2015, the FDA also approved Nivolumab for non-small-cell lung cancer (NSCLC)<sup>66-69</sup>. Both agents are monoclonal antibodies that inhibit the PD-1 receptor and its interaction with ligands. These antibodies have also shown benefits in patients with other advanced cancers<sup>70, 71</sup>.

The first large phase III trial of Nivolumab and Ipilimumab in GBM patients at different stages of treatment was initiated in 2014 (NCT02017717). Moreover, other PD-1/PD-L1 checkpoint inhibitors are under investigation for solid tumors and hematological cancers (AMP-224, MEDI4736, MPDL3280A, MSB0010418C)<sup>72</sup>.

TIM-3 and LAG-3 are also being investigated as possible targets for GBM therapy. TIM-3 is a glycoprotein with extracellular immunoglobulin and mucin domains that binds Galectin-9 and regulates T-cell exhaustion<sup>73</sup>. It has been shown to be highly expressed in patients with melanoma, NSCLC, lymphoma and other malignancies<sup>74</sup>. In recent investigations it was found that not only was TIM-3 expression significantly increased in peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells of glioma patients compared with healthy controls, but that the expression of galectin-9 in tumor tissues was associated with TIM-3 expression on Tumor Infiltrating Lymphocytes (TILs) and the WHO grade of glioma<sup>46, 75</sup>. Some studies show that combination therapy with anti-PD-1, anti-TIM-3 and focal radiation results in regression of murine gliomas, concluding that the addition of a second checkpoint-blocking antibody could achieve additive or synergistic antitumor effects<sup>76</sup>. Checkpoint blockade could also prove a useful adjuvant when combined with other therapeutic strategies. Indoleamine 2, 3 dioxygenase 1 (IDO) is a tryptophan catabolic enzyme induced in GBM patients<sup>77</sup>. IDO expression by brain tumor cells is critically important in the immunosuppressive tumor microenvironment because it modulates Treg activation, expansion and recruitment<sup>78</sup>. Recent research shows that blockade of CTLA-4 and PD-L1 in combination with IDO inhibition using 1-methyltryptophan (1-MT) in murine glioblastoma models results in a highly effective survival advantage and a significantly decreased level of Tregs, in comparison with the mono or dual therapies<sup>79</sup>.

LAG-3 is a CD4 homolog that binds MHC Class II<sup>80</sup> and seems to act synergistically with PD-L1 to control the expansion of already activated T-cells. It was found to be highly expressed on TILs from patients with melanoma, colorectal cancer or fibrosarcoma<sup>81</sup>. Currently, there is an ongoing phase I trial that studies the treatment with anti-LAG-3 mAb alone or in combination with Nivolumab in patients with recurrent glioblastoma (NCT02658981).

Of note, inhibition of immunological checkpoints not only improves the efficacy of immune-stimulant strategies, but can also be used in combination with conventional therapies, such as chemotherapy and radiotherapy<sup>16</sup>. Checkpoint inhibitors have also been shown to enhance the efficacy of anti-glioma stem cell treatments<sup>82</sup>.

## **2.2 Gene therapy**

Gene therapy initially developed to restore the function of defective or absent genes, and has gained popularity for the treatment of glioblastoma, due in part to the failure of other treatment options and to the successful design of many vectors able to safely deliver therapeutic genes into the tumor, such as viruses (adeno- and adeno-associated, retroviruses, herpes simplex virus, measles, reovirus, poliovirus), stem cells (neural, mesenchymal or embryonic), liposomes and nanoparticles<sup>17, 83</sup>. Current gene therapeutic approaches for glioma can be classified in four categories: suicide gene therapy, oncolytic virotherapy, tumor suppressor gene therapy and immuno-stimulatory therapy<sup>84</sup>. Viral mediated gene therapies can induce immunogenic cell death of malignant cells<sup>85</sup> and release of damage associated molecular patterns like HMGB1<sup>86, 87</sup>, leading to improved antigen presentation and enhanced anti-tumor immune response.

### **2.2.1 Suicide gene therapy**

Suicide gene therapy relies on the targeted administration of an enzyme, most commonly the viral gene thymidine kinase (TK), into dividing tumor cells. Upon administration of an inactive prodrug, for example ganciclovir (GCV), TK will convert it into a toxic metabolite, resulting in tumor cell apoptosis<sup>88-90</sup>. This strategy has shown success in numerous preclinical trials of glioma and has also been tested in several phase I and II clinical trials, which demonstrated the safety for this gene therapeutic approach<sup>91, 92</sup>. However, a large randomized phase III trial of HSV-TK/GCV in combination with surgery and radiation failed to show improved survival in newly diagnosed GBM<sup>93</sup>.

To improve upon this treatment, our group has developed a therapeutic strategy using adenovirus-mediated delivery of Flt3L, a cytokine which induces the generation and stimulates the migration of dendritic cells (DCs)<sup>94</sup>. In several preclinical studies we have shown that Ad-Flt3L in combination with AdTK/GCV induced prolonged survival of tumor-bearing animals, and that this was associated with increased recruitment of DCs into the tumor, generation of tumor specific T-cell responses and of long-term immunological memory, an effect dependent on the TLR2 activation by HMGB1 released by the dying tumor cells<sup>87, 95-99</sup>. A phase I clinical trial using this gene therapeutic approach is currently underway (NCT01811992, <https://clinicaltrials.gov/show/NCT01811992>).

### **2.2.2 Oncolytic viral therapy**

Oncolytic viral (OV) therapy exploits the ability of conditionally replicative viral vectors to selectively divide within tumor cells, induce their lysis, and amplify engineered therapeutic genes within the tumor environment. In addition to the direct oncolytic activity, OVs are also very effective at inducing immune responses to themselves and to the infected tumor cells<sup>85</sup>.

Oncolytic viruses tested in clinical trials for glioma include: oncolytic Herpes Simplex Virus (oHSV)<sup>100, 101</sup>, conditionally replicating adenoviruses (CRAds), oncolytic measles<sup>102</sup> and reovirus vectors<sup>103</sup>. These vectors have shown safety when administered in phase I clinical trials and show improvement over non-replicating viruses in terms of efficiency of transduction of the therapeutic gene. A drawback however is represented by the vector-induced immune response. Further modifying these vectors can reduce their antigenic properties, and efforts have been made to increase selectivity of the vectors, using glioma specific and radiotherapy inducible promoters, like survivin<sup>104, 105</sup>. Two CRAd's have been evaluated in clinical trials for glioma: ONYX-015, engineered to replicate in p53 deficient tumor cells, has been demonstrated to be safe when administered intracranially, but shows no clinical benefit<sup>106, 107</sup>, and Ad5Delta24, further modified into Ad5Delta24-RGD and DNX2401<sup>108</sup> to increase its targeting to tumor cells, is currently tested in several Phase I/II trials: NCT01582516- phase I/II trial testing the convection enhanced delivery of Delta-24rgd in patients with recurrent GBM, NCT02197169 – phase I unicentric trial (Target-I) testing DNX2401 expressing IFN- $\gamma$  in recurrent glioblastoma or gliosarcoma, and NCT01956734, a phase I trial testing DNX2401 in combination with temozolomide.

### **2.2.3 Immunomodulatory gene therapy**

Immunomodulatory gene therapy is designed to create a tumor environment optimized for the induction of an effective anti-tumor immune response. This has been tried with targeted delivery of cytokines into the tumor microenvironment to achieve higher local concentrations of immuno-stimulatory molecules, such as IL-2, IL-4, IL-12, IFN- $\gamma$  and IFN- $\beta$ , without generating systemic side effects.

IL-2, known to induce proliferation of all immune cells and to enhance production of other immunostimulatory cytokines has first been tested in glioma patients in 1986 when recombinant IL-2 and autologous lymphokine activated killer cells (LAK cells) were directly injected into the tumor cavity, demonstrating safety and in vitro tumor specific killing by LAKs<sup>109</sup>. Systemic administration of IL-2 has shown to be poorly tolerated<sup>110</sup>. In a phase I trial, combination of suicide gene therapy and IL-2, obtained through the administration of retroviral producing cells expressing IL-2 and HSV-TK resulted in minimal side effects and partial response, evidenced by MRI, in 2 of 12 patients<sup>111</sup>.

IL-4 stimulates proliferation of lymphocytes acting synergistically with IL-2 and also induces DC maturation and production of IL-12. In a rat glioma model, subcutaneous vaccines with 9L cells transduced with HSV expressing TK, IL-4, or TK + IL-4 inhibited subcutaneous tumor growth and increased survival upon challenge with intracranial 9L tumors<sup>112</sup>. This was correlated with increased production of IFN- $\gamma$  in the splenocytes of treated animals challenged with irradiated tumor cells. This strategy was tested in a phase I trial<sup>113</sup>, results of which have not been published.

The type I interferon gene IFN- $\beta$  has been widely tested in treatments of several cancers, showing direct antiproliferative effect, and in glioma enhances sensitivity for TMZ treatment<sup>114</sup>.<sup>115</sup> In Japan, a phase I clinical trial with liposomal delivery of IFN- $\beta$  showed minimal toxicity and clinical response with more than 50% reduction of the tumor in 2 out of 5 patients<sup>116</sup>. This study was followed by another phase I trial using administration of Ad-hIFN $\beta$  into the tumor cavity and surrounding tumor area post resection, demonstrating safety and induction of tumor cell apoptosis<sup>117</sup>.

## 2.3 Active immunotherapy

Cancer vaccines aim at stimulating adaptive immune responses that target tumor-specific antigens. Strategies utilized include delivery of tumor-associated antigens, administration of tumor antigen loaded DCs and tumor cell vaccines.

### 2.3.1 Peptide vaccination: tumor associated antigens and shared neoantigens

Successful immunotherapies rely on the targeting of glioma-specific antigens to selectively kill tumor cells, without inducing autoimmune reactions. To date, numerous glioma associated antigens (GAA) have been identified (**Table 2**), and targeted therapies have been developed and tested in recently completed and ongoing clinical trials. Several known tumor-associated antigens such as HER-2, TRP-2, gp100, MAGE-1, IL-13 $\alpha$ 2 and AIM-2 are being targeted in GBM<sup>16-18</sup>. Additionally, new immuno-therapeutic strategies use neoantigens to specifically target tumor cells. Neoantigens represent antigenic molecules, absent from normal tissues, which result from tumor specific DNA somatic mutations present within protein-coding regions; commonly occurring mutations, shared by many tumors, are attractive targets for therapy<sup>118, 119</sup>. The most extensively studied target is EGFR variant III<sup>16-18</sup>. EGFRvIII is a mutant form of the EGFR gene, found in 20–30% of GBM patients, expressing a truncated, constitutively active form of the receptor, causing increased proliferation and survival advantage of GBM tumor cells<sup>120</sup>. Evaluation of Rindopepimut/CDX-110 (14 amino acid peptide conjugated to KLH) in 18 patients with EGFRvIII mutation in combination with standard radiotherapy and chemotherapy showed a median survival of 26 months. At recurrence 82% of the tumors lost EGFRvIII expression suggesting treatment induced immunoediting. Currently a phase III (NCT01480479) multi-center trial is looking at the efficacy of CDX-110, GMCSF,

TMZ and keyhole limpet hemocyanin (KLH) for the treatment of adult patients with EGFRvIII positive gliomas and a phase II study is evaluating the combination of rindopepimut, GM-CSF and bevacizumab in relapsed EGFRvIII positive gliomas (NCT01498328).

Mutations of IDH1 and IDH2 have been found in more than 80% of WHO grade II/III astrocytomas, oligodendrogliomas and oligoastrocytomas<sup>5</sup>. For IDH1, the most common genetic alteration is represented by a single nucleotide substitution: R132H, encompassing more than 70% of all IDH mutations. To test if this neoantigen is amenable for immunotherapeutic targeting, Schumacher et al.<sup>121</sup> generated a peptide library encompassing this mutation and tested its ability to bind MHC-I and MHC-II. This study demonstrates that several 15mer peptides bound to the human MHC-II allele HLA-DRB1\*0101 and that MHC-II humanized mice immunized with one of these peptides developed robust IFN $\gamma$  T-cell responses resulting in Th1 CD4 specific responses with generation of mutation-specific anti-IDH1 antibodies detectable in the serum. Furthermore, specific IFN $\gamma$  producing T cells were isolated from the serum of patients harboring IDH1 (R132H)-mutated gliomas but not from patients with wild type IDH gliomas. Also tumor growth was reduced in MHC-II humanized mice immunized with the IDH1 (R132H) peptide vaccine, effect dependent on CD4<sup>+</sup> cells. Notably, immunization with the mutant peptide induced immuno-editing of the IDH1 (R132H) tumors, which resisted vaccination and showed marked decrease in IDH1 (R132H) expression. This study highlights the importance of CD4<sup>+</sup> T-cell-mediated anti-tumor immune response in the absence of an MHC-class-I-restricted CD8<sup>+</sup> T cell response and the potential therapeutic success when targeting this neoantigen with immunotherapy. Currently, an open clinical trial (RESIST, led by Dr. Vlahovicat at Duke University, NCT02193347) is testing the use of the IDH1 Peptide Vaccine (PEPIDH1M) administered with GM-CSF and tetanus toxoid preconditioning of the vaccine site



in adult patients with recurrent grade II glioma positive for IDH1R132H. Another Phase 1 clinical trial (NOA-16) is testing an IDH1 Peptide Vaccine in patients with IDH1R132H mutant grade II and IV tumors with ATRX loss and without 1p/19q codeletion.

Diffuse intrinsic pontine glioma (DIPG) is often associated (70%) with missense mutation of the lysine in the histone H3.3 K27M, with the mutation also present in 30% of adult GBM patients. A recent report tested whether this mutation can serve as neoantigen for immunotherapy and if it can initiate a specific CTL response. Preliminary results show that out of 4 peptides generated by a peptide–MHC binding algorithm and a proteosomal cleavage site prediction system, one was able to induce specific CTL responses which specifically lysed HLA-A2<sup>+</sup> DIPG cell lines harboring the mutation<sup>122</sup>. If proven safe and effective this therapeutic strategy will bring great hope for children with DIPG.

One potential drawback of single peptide vaccination is immuno-editing; the concept of immuno-editing, describing the crosstalk between the immune system and cancer, has developed over the last century from the idea of immune surveillance, which posits that the immune system is continuously monitoring and capable of eliminating cells displaying neoplastic mutations<sup>123-125</sup>. As cancer still develops in immuno-competent organisms, the immune surveillance is eventually overcome due to the aggressive growth and invasion of cancers and to immuno-suppressive mechanisms induced by malignancy<sup>126</sup>. Immuno-editing represents a major obstacle for the success of immunotherapies for glioma. Deciphering mechanisms of immuno-editing will lead to an increased understanding of glioma pathogenesis and progression and to the design of more successful immunotherapies for this disease.

As discussed above, an illustrative example of glioma immuno-editing following targeted

immune therapy is represented by the recently completed Phase II multicenter study (ACTIVATE, ACTII), which used the CDX-110 vaccine (Rindopepimut) concurrent with temozolomide in patients with newly diagnosed EGFRvIII-positive GBM<sup>127</sup>. The ACTII trial demonstrated that OS was positively correlated with the development of specific antibody responses against EGFRvIII (identified in 6 of 14 patients), and that remarkably, at recurrence 82% of patients had lost EGFRvIII expression<sup>127, 128</sup>. Immuno-editing was also demonstrated in a subsequent Phase II multicenter single-arm trial (ACTIII) using the same therapeutic approach, showing decrease of EGFRvIII immunoreactivity in 67% (4 of 6 patients), and an OS of 21.8 months<sup>129</sup>. A Phase III Study (ACT IV) of Rindopepimut, GM-CSF, TMZ and KLH for patients with newly diagnosed EGFRvIII positive glioblastoma will likely be discontinued as the interim analysis showed that the treatment is unlikely to meet statistically significant primary OS endpoint in patients with minimal residual disease<sup>130</sup>.

To reduce the risk of immuno-editing, immune tolerance and disease recurrence following single peptide vaccinations, many studies are investigating the production of effective combinations of several GAA. A recently completed study has tested the intradermal administration of a combination of three peptides: EphA2, IL-13R $\alpha$ 2 and survivin together with tetanus toxoid and poly [I:C] as adjuvants in pediatric brain stem and high grade gliomas (NCT01130077). Results showed that the vaccine was well tolerated, induced the development of specific GAA immune responses, and led to favorable clinical outcome, particularly in patients manifesting temporary pseudo-progression, indicative of immune cell infiltration within the tumors causing edema<sup>131</sup>. Other ongoing clinical trials are testing a combination of the immunogenic peptides IL13R $\alpha$ 2, EphA2 and Survivin (SL-701 vaccine in combination with bevacizumab, NCT02078648) in patients with newly diagnosed GBM. The ICT-107 vaccine is

an autologous DC vaccine pulsed with six peptides (HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2 and AIM-2). In the initial phase I trial this treatment induced a response in 33% of the 21 patients enrolled with a median OS of 38.4 months<sup>132</sup>. This vaccine is currently being tested in a randomized, two-arm, phase III study with ICT-107 or placebo control in combination with the SOC (NCT02546102). Ongoing clinical trials are testing a proprietary combination of 11 HLA-A2 restricted tumor-associated peptides IMA950 alone (NCT020278648) or in combination with GMCSF (NCT01222221) or TLR3 agonist, Poly ICLC (NCT01920191).

### **2.3.2 Peptide vaccination: patient-specific neoantigens**

Identification of patient-specific neoantigens has been made possible by the advent of next-generation DNA and RNA deep sequencing technology, the development of prediction algorithms, MHC multimer based screens<sup>133</sup>, and high throughput sequencing of antigen-specific receptors of whole lymphocyte populations (TCRseq, BCRseq) capable of identifying patient-specific combinations of immunogenic neo-antigens. That specific T cell reactivity can be induced by neoantigens identified through cancer exome sequencing has been demonstrated in melanoma patients<sup>134, 135</sup>, results which prompted the development of clinical trials to target patient-specific neoantigens with immune therapy in several cancers. This strategy has been adopted by the European Glioma Actively Personalized Vaccine (GAPVAC) Consortium in a phase I clinical trial (NCT02149225) testing personalized peptide vaccines in combination with Poly-ICLC and GM-CSF in newly diagnosed glioblastoma. A phase-I clinical trial led by Dr. Reardon at the Dana Farber Cancer Institute is testing the safety and efficacy of neoantigen based vaccines in combination with radiation in MGMT-unmethylated newly-diagnosed GBM (NCT02287428).

An avenue of increased interest in the management of cancers is represented by the

targeting of neoantigens induced by radiation therapy<sup>136, 137</sup>. Ionizing radiation induces DNA strand breaks, which will kill rapidly dividing cells and induce immunogenic cell death, generating damage associated molecular patterns, like HMGB1<sup>138</sup> and induce phenotypic changes in normal and malignant cells. It has been shown in preclinical models of glioma that radiation therapy induces the expression of novel targets such as integrins  $\alpha\beta3$  and matrix metalloproteinases MMP2 and MMP9, which can promote glioma progression<sup>139</sup>, however when combined with immune checkpoint blockade using anti PD-1 antibodies, radiation therapy induces long-term survival in glioma bearing animals<sup>140</sup>. In other cancer models it has also been shown that radiation induces various cell surface receptors on tumor cells (MHC-I, ICAM-1, Fas), and that radiotherapy augmented tumor cell killing by antigen specific T cells<sup>136</sup>. Combining next generation sequencing with radiotherapy to identify immunogenic mutations<sup>141</sup> and designing therapeutic antigenic peptides to be used as agents in combinatorial approaches with radiation and immune checkpoint blockade are attractive strategies to pursue further for the treatment of glioblastoma.

A recent study from our lab has shown that loss of ATRX increases genetic instability. Furthermore, analysis of genome-wide data for human gliomas showed that ATRX mutation is associated with increased mutation rate at the single-nucleotide variant (SNV) level<sup>8</sup>. Tumors with ATRX loss could therefore potentially be more immunogenic due to the generation of neoepitopes or neoantigens. As pointed out by Schumacher et al, such neoantigens are crucial to tumor rejection because the T cell repertoire against these antigens is not affected by central tolerance<sup>118</sup>.

### 2.3.3 DC Vaccination

DCs are the most efficient APCs that can activate CD4, CD8, NK and NKT cells<sup>17</sup>.

Factors that can modulate the efficacy of DC vaccination strategies include methods of DC differentiation, loading with tumor antigens, route of administration, and adjuvants used (**Fig 2 and Table 3**)<sup>17</sup>. The most advanced current clinical trial using an autologous DC vaccine-DCVax-L is in Phase III (NCT00045968). While the vaccine was shown to be safe, limited clinical benefit was observed. Combination of autologous DC vaccine with Imiquimod or Poly ICLC showed increased OS in patients with mesenchymal gene signature<sup>142</sup>. Increased OS was also seen in 33% of the patients treated with DC vaccine pulsed with six GAA peptides<sup>132</sup>. Treatment of 22 patients with recurrent GBM with  $\alpha$ -type 1 polarized DCs pulsed with EphA2, IL13R $\alpha$ 2, YKL-40 and gp100 combined with poly ICLC showed the induction of a positive immune response in 58% of the patients<sup>143</sup>. An interesting approach using DC vaccination was utilized to target glioma stem cells (GSCs). A recently completed phase I/II trial (NCT00846456) using DCs transfected with autologous GSC mRNA demonstrated significantly longer PFS and OS than the controls. Like EGFRvIII peptide vaccines, DC vaccines pulsed with CDX-110 showed safety and efficacy in eliciting an anti-tumor immune response and improved survival in GBM patients that expressed the variant<sup>128</sup>. Results from a randomized and blinded clinical trial by the Sampson group have demonstrated that pre-conditioning the vaccine site with antigens such as tetanus/diphtheria toxoid can significantly improve the efficacy of tumor-antigen-specific DCs<sup>144</sup>. Patients given tetanus toxoid had enhanced DC migration bilaterally and significantly improved survival<sup>144</sup>.

#### **2.3.4 Tumor cell vaccination**

Encouraging results were seen in a prospective Phase I/II clinical trial using temozolomide, radiotherapy and autologous formalin fixed tumor cell vaccine in 24 patients with newly

diagnosed GBM<sup>145</sup>. A recent phase II clinical trial of adult recurrent GBM in which vaccines were made by isolating tumor gp96 complexes from autologous-resected tumors (HSPPC-96) demonstrated the safety and OS of 42.6 weeks<sup>146</sup>. A randomized phase II trial will compare the efficacy of HSPPC-96 vaccine with or without bevacizumab in recurrent resectable GBMs (NCT01814813).

### **2.3.5 Immune stimulatory adjuvants**

Immune adjuvants can enhance vaccine associated anti-tumor immunity through mechanisms such as antigen clustering, maintaining an antigen depot, stimulating innate immunity, promoting DC maturation, and blocking Treg induced suppression<sup>16-18</sup>. Compounds most commonly evaluated as adjuvants are poly I:C, CpG oligonucleotides, and Imiquimod that activate TLR3, TLR9 and TLR7 respectively<sup>16-18</sup>. TLRs are pathogen associated molecular pattern receptors that when activated increase the production of immune-stimulatory cytokines and costimulatory molecule expression on APCs<sup>147</sup>. In a prospective phase II clinical trial of pediatric glioma, treatment with poly ICLC was well tolerated by children. 5 out of 10 children showed long-term stable disease<sup>148</sup>. Another phase II trial testing the combination of poly ICLC with radiotherapy and TMZ concluded that poly ICLC administration may improve the efficacy of radiotherapy and TMZ treatment with no added adverse effects<sup>149</sup>. While CpG-ODN showed promise in many preclinical and phase I clinical studies, intratumoral administration of CpG ODN as a single treatment modality showed little benefit in a phase II clinical study with patients with recurrent GBM<sup>150</sup>. Besides TLR agonists, other immune stimulatory adjuvants have also been tested. Administration of tetanus toxoid was shown to improve the efficacy of DC vaccination in GBM patients and to prolong their survival<sup>144</sup>. Mitchell et al. reported that tetanus toxoid preconditioning in vaccination site in a mouse glioma model enhanced DC migration and

suppressed tumor growth in a CCL3 dependent fashion<sup>144</sup>. KLH is being tested in active vaccination studies for GBM patients. GMCSF has also been used to promote vaccine immunogenicity by enhancing DC maturation<sup>151</sup>.

#### **2.4 Passive immunotherapy: antibodies**

Monoclonal antibody therapy results in tumor cell death through a variety of immune and non-immune mediated mechanisms<sup>152</sup>. Ideally the expression of the antigen recognized by the antibody would be restricted to the tumor cells, so as to minimize injury to normal tissues. GBMs are highly vascular tumors that produce large amounts of VEGF. Currently, the most prominent VEGF targeting drug is bevacizumab (BEV), a recombinant humanized monoclonal antibody that binds to human VEGF-A. Several phase II trials have tested the efficacy of anti-VEGF therapies either alone or in combination with irinotecan, etoposide, nitrosourea, or other agents<sup>153</sup>. While BEV proved to be a safe and feasible treatment option, the initially reported promising response rates might partly be attributed to imaging limitations resulting in an apparent but debatable reduction in the contrast-enhancing tumor volume [31, 32]. Recent prospective phase III trials (AVAglio & RTOG 0825) were designed to prove the efficacy of TMZ based radio chemotherapy in combination with BEV as first-line therapy for GBM. While the RTOG 0825 trial failed to show significant benefits in terms of progression free (PFS) and overall survival (OS), the AVAglio study demonstrated a significant prolongation of PFS by 4.4 months in the BEV arm. However, this PFS benefit did not translate into an improvement in OS<sup>153</sup>. Epidermal growth factor receptor gene mutation is a frequent finding in GBM with EGFR variant VIII being the most common one. Combination therapy of anti-EGFRvIII antibody, cetuximab in combination with bevacizumab/irinotecan was not found to be superior than bevacizumab/irinotecan alone. Intra-arterial infusion of cetuximab alone or in combination with

BEV is currently being tested<sup>154</sup> (NCT01884740 and NCT01238237). Another antibody against EGFR, nimotuzumab, in combination with radiation and chemotherapy in patients with diffuse intrinsic pontine gliomas has shown an increased median OS of 15 months when compared to 9.4 months for nimotuzumab and radiation alone<sup>155</sup>. A randomised, open label phase III trial was conducted to evaluate efficacy of nimotuzumab added to standard therapy for newly diagnosed glioblastoma. No statistically significant increase in overall survival was observed by the addition of nimotuzumab<sup>156</sup>. ABT-414 is an antibody-drug conjugate that delivers Monomethyl Auristatin F or MMAF to cells with active EGFR or mutant EGFRvIII. Interim results from a phase I clinical trial testing ABT 414 as a monotherapy, in combination with chemotherapy and in combination with radiation and chemotherapy showed objective responses in 4 out of 12 patients, including 2 that showed complete response (NCT02573324).

## **2.5 Adoptive immunotherapy: autologous T cell transfer and CAR T cells**

Adoptive T cell transfer involves directly transferring T cells with high avidity against the tumor antigens to the host to generate anti-tumor immunity<sup>152</sup>. Rosenberg et al pioneered the technique in a subset of melanoma patients by expanding TILs obtained from resected melanoma tumors<sup>157</sup>. The technique however was limited by the availability of tumor-specific lymphocytes. To overcome this limitation, the same group developed patient T cells genetically modified to express a T cell receptor that recognized melanoma antigen MART-1, resulting in tumor regression<sup>158</sup>. Early approaches utilizing adoptive transfer for glioma involved administration of T cells isolated from dLNs following s.c. injection of irradiated tumor cells with GM-CSF or *ex vivo* expansion of T cells induced by culture with tumor cells<sup>152</sup>. In a phase I clinical trial in patients with recurrent GBM and cytomegalovirus positive serology, adoptive transfer of autologous CMV-peptide expanded T cells resulted in a PFS of 243 days in 4 out of 10



patients<sup>159</sup>. Adoptive T cell transfers are restricted by the need to match the HLA type, and next generation T cell transfer strategies use CAR T cells. Chimeric antigen receptor T cells, or CAR T cells, are engineered to target a specific antigen by combining the recognition specificity of an antibody with T cell signaling through the CD3  $\zeta$  chain or FC $\epsilon$ RI $\gamma$ <sup>160</sup>. A serious concern with the use of this approach is the damage that can occur to normal tissues if the antigen expression is not tumor specific. Thus it is essential to select targets that show tumor restricted expression. Using CARs as a therapeutic strategy in brain tumors was first tested by the Jensen group, who showed that intratumoral delivery of IL-13 zetakine CAR eliminated orthotopic human glioma tumors in immune compromised mice<sup>161</sup>. The clinical trial testing the safety and feasibility of this therapy in patients with recurrent GBM has shown minimal side effects, and 2 out of 3 patients who received repeated intracranial infusions of IL-13 zetakine+ CTLs showed transient anti-glioma immune responses<sup>162</sup>. HER2-specific CAR T cells have been shown to generate a HER2-dependent antitumor response with increased production of IFN- $\gamma$  and IL-2, resulting in tumor regression in a xenograft mouse GBM model<sup>163</sup>. A phase I trial will test the safety and efficacy of using HER2-specific CARs in patients with recurrent GBM (NCT02442297). The Rosenberg group at NCI (NCT01454596) and the University of Pennsylvania/Novartis (NCT02209376) are also currently recruiting participants to test the safety and feasibility of administering T cells expressing anti-EGFRvIII CAR to patients with gliomas expressing EGFRvIII. The role of engineered T cell therapy may be expanded in the future to include gene transfer mediating extended survival, enhanced tumor penetration, or resistance to immunosuppression<sup>164</sup>.

### 3. Predictive biomarkers for immunotherapy

Significant advances have been made in genetic and molecular characterization of tumors, allowing for the identification of predictive biomarkers for glioma immunotherapy. As mentioned above, clinical trials of EGFRvIII CAR T cell therapy (NCT02209376) and EGFR peptide vaccination (NCT00458601, NCT01480479) used glioma EGFRvIII expression as a precondition for enrollment, limiting the potential for adverse events in those who were not likely to experience therapeutic benefit<sup>165</sup>. Similarly, IL-13R $\alpha$ 2 expression was used as a precondition for enrollment in a clinical trial of IL-13R $\alpha$ 2 CAR T cells (NCT02208362). Other biomarkers such as IDH1/2 mutation, 1p/10q deletion, MGMT methylation, ATRX loss, and H3.3 K27M mutation<sup>166</sup> are prognostically useful, and their ability to predict therapeutic efficacy of a particular immunotherapy remains open for exploration<sup>167</sup>. Large scale clustering of several biomarkers into neural, proneural, classical, and mesenchymal subtypes corresponds with differences in treatment efficacy of chemotherapy and radiotherapy, and holds significant potential for informing efficacy of immunotherapy<sup>168</sup>. In a Phase I trial testing autologous tumor lysate-pulsed DC vaccination (NCT01204684), the study population was stratified according to the genetic expression signature of tumor cells (mesenchymal, proneural and proliferative), and patients with mesenchymal signatures, the worst prognostic subtype, had significantly extended survival compared to a historical control cohort with the same signature, whereas no survival difference was observed in those with a proneural gene expression signature<sup>142</sup>. Moreover, the mesenchymal subtype showed higher numbers of CD3+ and CD8+ TILs compared with other subtypes, suggesting that gene expression profile can be used as a biomarker for choosing an efficacious immunotherapy.

Alternatively, biomarkers which are not tumor-associated may also hold significant predictive value. Hsu et al. analyzed sequence similarity of the T cell receptor (TCR)  $\beta$  chain

gene between T cells in the tumor and those in the peripheral blood. They showed that two patients with the highest TCR sequence similarity between tumor and blood before treatment showed the longest survival among 5 patients who were treated with DC vaccine<sup>169</sup>. HLA type may be an important predictive biomarker for peptide vaccines. The EphA2, IL-13R $\alpha$ 2, and survivin peptide vaccine was optimized to bind HLA-A2 most efficiently; thus the clinical trial prescreened for HLA-A2 expression prior to enrollment (NCT01130077). Humoral biomarkers may also aid in monitoring biological effects of immunotherapy. Recently, Everson et al. tested the association of phospho-STAT signaling and patient survival after DC vaccine treatment<sup>170</sup>. An increased per cell phosphorylation of STAT-5 in cytotoxic T-cells following IL-2 stimulation was associated with extended survival (log rank  $p = 0.0015$ ). Patients who survived longer than two years had a significantly greater phosphorylated STAT-5 ratio ( $p = 0.015$ ).

#### **4. Expert opinion**

GBMs remain one of the most difficult to treat human cancers. In spite of optimal standard of care, delivered in the most complex and advanced medical institutions, its prognosis has remained dismal, with a median survival of ~12-18 months post diagnosis<sup>9, 16, 18</sup>. This is thought to be due to the fact that GBMs are usually diagnosed at late stages of the disease; the blood brain barrier precludes the effective action of chemotherapeutic agents, there is a paucity of antigen presenting cells (APCs) within the central nervous system, and GBM is highly immunosuppressive<sup>30, 31, 33, 39, 45, 49, 171</sup>. The tumor microenvironment in GBMs is composed mainly of immunosuppressive cells, i.e., regulatory T cells (Tregs), tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDCs). These cells exhibit immunosuppressive effects, which inhibit anti-tumor specific cytotoxic T cells functions, ultimately leading to decreased efficacy of immunotherapeutic approaches. In addition, GBMs

are highly heterogeneous, with tumor cells acquiring new mutations after treatment, leading to therapy resistant disease<sup>172, 173</sup>.

In GBM, tumor infiltrating T cells and Tregs exhibit high levels of the immunosuppressive receptor, CTLA4. CTLA4 binds with high affinity to CD80 and CD86, which are present on APCs, therefore inhibiting their binding to the T cells' immune stimulatory receptor CD28<sup>22, 43, 72</sup>. This powerful immunosuppressive mechanism can be overcome by the use of antibodies which block CTLA4<sup>22, 43, 72</sup>. Another powerful immunosuppressive mechanism is mediated by PD1-PDL1/PDL2 interactions, which occur during the effector phase of T cells functions leading to apoptosis of cytotoxic T cells<sup>22, 43, 72</sup>. Thus, immune checkpoint blockade has become a very attractive therapeutic target to be used in combination with other chemotherapeutic strategies, radiation, and/or immunotherapies<sup>22, 72, 174</sup>. Although these approaches are yielding very promising results in GBM preclinical models<sup>62, 76</sup> and clinically for the treatment of immunogenic cancers, such as melanoma<sup>56, 66</sup>, no data is available yet in relation to their clinical efficacy in GBM. The clinical trial approach that we developed in our laboratory, which entails using a combination of a conditional cytotoxic gene therapy approach<sup>88, 89</sup> together with an immune stimulatory approach to attract APCs to the tumor microenvironment<sup>87, 95-99</sup>, has proven to be highly efficacious in several rat and mouse syngeneic, intracranial GBM models and is currently being tested in the clinic (<https://clinicaltrials.gov/show/NCT01811992>). In summary, taking into account the formidable challenges posed by GBM, we hypothesize that in order to elicit therapeutic benefit, it will be necessary to use combination therapies. These should include, surgery (aiming to perform aggressive resections when clinically possible) chemotherapy, radiation, and immune stimulatory approaches. These could include vaccination strategies, intra-tumor gene therapy strategies, oncolytic virotherapy, and immune checkpoint

blockade. Although we have a long way ahead, we believe that the basic science discoveries related to how the immune system works in GBM, coupled with well-designed clinical trials, will enable the scientific/medical community to make inroads into developing novel combination therapies that will elicit improved median survival and better prognosis for this devastating cancer.

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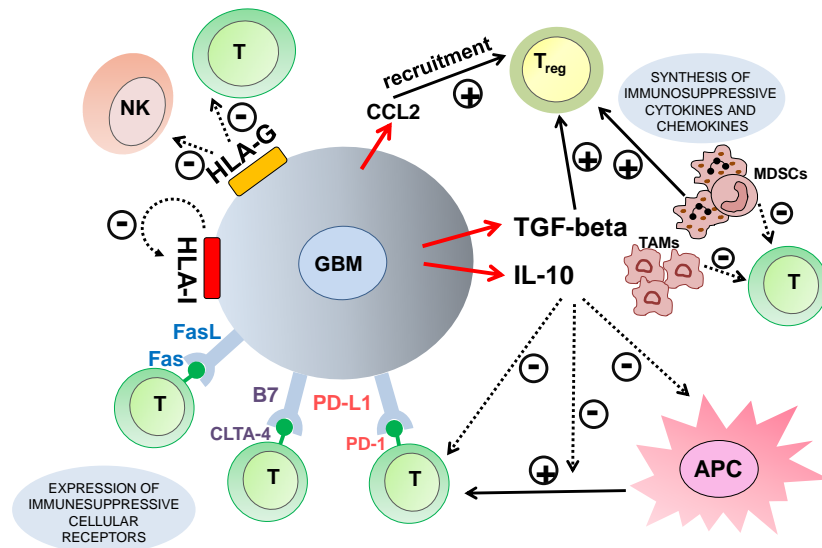
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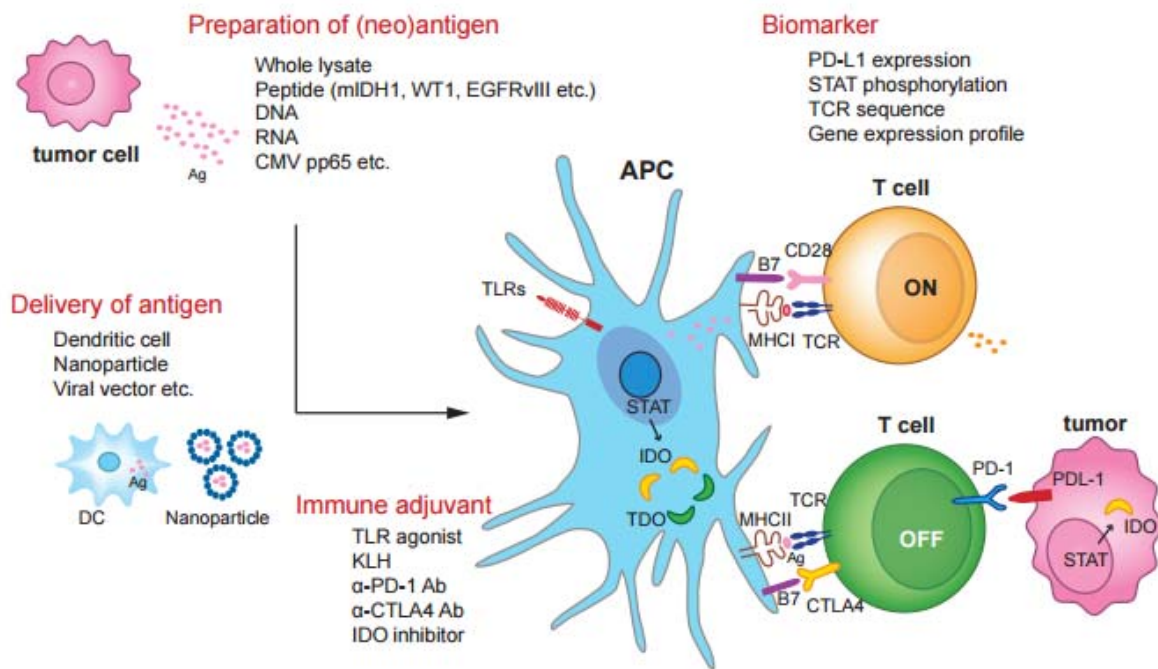
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**Figure 1: Role of GBM cells in the generation and maintainance of the immunosuppressive tumor microenvironment.** GBM cells have a central role in the immunosuppressive nature of tumor microenvironment. GBM cells express membrane-bound and soluble immunosuppressive proteins. FasL induces apoptosis of tumor infiltrating lymphocytes. B7 molecules activate CTLA-4 and PD-L1 are ligands of immunosuppressive receptors CTLA-4 and PD-1, which inhibit effector T cells that infiltrate the tumor microenvironment. Downregulation of HLA-I reduces the immunogenicity of GBM cells, which in turn could activate NK cell response against the tumor. However, GBM cells overexpress an alternative MHC I molecule, HLA-G, which inhibits NK and CTL response. The synthesis of antiinflammatory cytokines and chemokines also contribute to the immunosuppressive microenvironment. GBM cells produce CCL2 and TGF-beta and IL-10 which recruit Tregs, myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) that in turn inhibit the T cell response. IL-10 also inhibits the function of antigen presenting cells (APCs) within the tumor.



**Figure 2: Schematic showing factors that impact vaccination strategy for glioblastoma:** (1) preparation and (2) delivery of tumor associated antigens, (3) immune adjuvant to boost immune reactions against tumor antigens and (4) biomarkers to precisely estimate the efficacy of vaccination and to select proper patient populations. Immune adjuvants are divided in two groups: (1) agonists for pro-inflammatory molecules such as TLR agonists or ligation of B7 and CD28 receptors, and (2) antagonists for anti-inflammatory molecules such as anti-PD-1 blocking antibodies or IDO inhibitors. DC represents dendritic cell; CMV, cytomegalovirus; APC, antigen presenting cell, TLR, toll like receptor.



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**Table 1: Overview of molecular and genetic alterations in glioma**

<b>Adult Primary</b>				
<b>Tumor Subtype</b>	<b>Classical</b>	<b>Mesenchymal</b>	<b>Neural</b>	<b>Proneural</b>
<b>overexpression</b>	<i>EGFR</i> , <i>NES</i> <sup>168, 175</sup>	<i>MET</i> , <i>CHI3LI</i> , <i>CD44</i> , <i>MERTK</i> <sup>175</sup>	EGFR(1,2)	<i>PDGFRA</i> <sup>168, 175</sup>
<b>low expression/ablation</b>	<i>PTEN</i> , <i>CDKN2A</i> <sup>168, 175</sup>	<i>NF1</i> , <i>PTEN</i> <sup>168, 175</sup>		<i>PTEN</i> <sup>175</sup>
<b>mutation</b>	<i>EGFR</i> <sup>168, 175</sup>	<i>NF1</i> <sup>168, 175</sup> , <i>PTEN</i> <sup>168</sup>		<i>IDH1</i> , <i>PIK3A/RI</i> <sup>168, 175</sup>
<b>pathway activity</b>	Notch <sup>168, 175</sup> , Sonic hedgehog <sup>168</sup>	TNF, NFκB <sup>168, 175</sup>		HIF, PI3K, PDGF <sup>175</sup>
<b>marker expression</b>		<i>CHI3LI</i> , <i>MET</i> <sup>168</sup>	<i>NEFL</i> , <i>GABRA1</i> , <i>SYT</i> , <i>SLCIA5</i> <sup>168, 175</sup>	<i>SOX</i> , <i>DCX</i> , <i>DLL3</i> , <i>ASCL1</i> , <i>TCF4</i> <sup>168, 175</sup>

<b>Tumor Type</b>	<b>Adult Secondary</b>	<b>Young Adult</b>
<b>overexpression</b>	<i>PDGFRA</i> <sup>175</sup>	
<b>low expression/ablation</b>	<i>PTEN</i> <sup>175, 176</sup>	<i>PTEN</i> <sup>176</sup>
<b>mutation</b>	<i>TP53</i> , <i>IDH1</i> <sup>175, 176</sup> , <i>ATRX</i> <sup>176, 177</sup>	<i>TP53</i> , <i>IDH1</i> <sup>176, 178</sup> , <i>H3.G34</i> <sup>178, 179</sup> , <i>ATRX</i> <sup>178-180</sup>
<b>pathway activity</b>	HIF, PI3K, PDGFRA <sup>175</sup>	
<b>marker expression</b>	<i>PDGFRA</i> , <i>OLIG2</i> , <i>TCF3</i> , <i>NKX2</i> <sup>175</sup>	



**Table 2: Glioma associated antigens as therapeutic targets**

Antigen	Description	Expression	Tested in clinical trials?
ABCG <sup>181</sup>	ATP – Binding Cassette Group	Glioma stem cells	N/A
Aim-2 <sup>29, 132, 182, 183</sup>	Antigen Isolated Melanoma-2	High grade astrocytoma	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2, and AIM-2 peptides <i>33% immune response</i> <sup>132</sup> ICT-107 DC Vaccine (NCT02546102) <i>ongoing</i>
Art1 <sup>29</sup>	Antigen Recognized by T cells-1	Adult and pediatric brain tumors	N/A
Art4 <sup>29</sup>	Antigen Recognized by T cells-4	Adult GBM	N/A
B-catenin <sup>184, 185</sup>	Beta-catenin	Astrocytomas	N/A
BMI1 <sup>186-188</sup>	polycomb-group (PcG) BMI1	Medulloblastoma, Glioblastoma, Glioma stem cells	N/A
B-cyclin <sup>29, 183, 189</sup>	B-cyclin	Adult GBM	N/A
Cathepsin B <sup>190-192</sup>	Cathepsin B	Glioma stem cells, glioblastoma	N/A
Cav-1 <sup>193, 194</sup>	Caveolin -1	Anaplastic astrocytoma, Glioblastoma, Oligodendroglioma, Ependymoma	N/A
CD133 <sup>195-198</sup>	Cluster of differentiation 133, Prominin	Glioma stem cells	ICT-121 DC vaccine pulsed with CD133 (NCT02049489) <i>ongoing</i>
CD74 <sup>199, 200</sup>	Cluster of Differentiation 74, HLA class II histocompatibility antigen	Glioblastoma	N/A

	gamma chain Binds Macrophage Migration Inhibitory Factor		
COX-2 <sup>201-203</sup>	Cyclo-oxygenase 2	Glioblastoma, CD133+ GSC cells	COX-2 inhibitor celecoxib and TMZ with thalidomide or isotretinoin (NCT00112502) <i>No difference in PFS or OS</i> <sup>202</sup>
E-Cadherin <sup>204, 205</sup>	Epithelial-calcium-dependent adhesion	Glioblastoma, Pontine glioma, Medulloblastoma	N/A
EGFRvIII <sup>50, 127-129, 196, 206</sup>	Epidermal growth factor receptor variant III (truncated, constitutively active form of the receptors)	Glioblastoma	<b>EGFRvIII peptide vaccine</b>  ACTIVATE, ACTII (NCT00643097) <i>43% EGFRvIII-specific antibody response</i> <sup>127</sup>  ACTIII (NCT00458601) <i>85% EGFRvIII-specific antibody response</i> <sup>129</sup>  ACTIV (NCT01480479) <i>ongoing</i>  ReACT (NCT01498328) <i>ongoing</i>  <b>EGFRvIII CARs</b>  NCT02209376 <i>ongoing</i>  NCT01454596 <i>ongoing</i>
Ephrin A2/Eck <sup>29, 143, 183, 207-210</sup>	Ephrin Receptor A2 /epithelial cell kinase	Glioma Stem Cells, Glioblastoma, Adult and pediatric brain tumors	DC vaccine pulsed with EphA2, IL-13R $\alpha$ 2, YKL-40, and gp100 (NCT00766753) <i>58% immune response</i> <sup>143</sup>  EphA2, IL-13R $\alpha$ 2, and survivin peptide vaccine (NCT01130077) <i>62% peptide specific</i>

			<i>immune response</i> <sup>131</sup> SL-701 peptide vaccine (NCT02078648) <i>ongoing</i>
EZH2 <sup>29, 209, 211</sup>	Enhancer of zeste homolog 2	Adult GBM, Glioma stem cells	N/A
Fra-1/Fosl 1 <sup>29, 207, 209</sup>	Fos related antigen 1	Adult GBM, highly expressed in pediatric brain tumors	N/A
GAGE-1 <sup>29, 212</sup>	G antigen 1	Low expression in adult high grade and pediatric brain tumors	N/A
Ganglioside/ GD2 <sup>213, 214</sup>	Ganglioside GD2	Astrocytic tumors	N/A
GLAST <sup>215, 216</sup>	GLutamate ASpartate Transporter, Solute carrier family 1 (glial high-affinity glutamate transporter), member 3, SLC1A3, Excitatory Amino Acid Transporter 1 (EAAT1) .	Glioma stem cells	N/A
Gli-1 <sup>217-219</sup>	Glioma-associated oncogene homolog 1	Glioblastoma, recurrent Glioblastoma after chemotherapy	IPI-926: Hedgehog pathway inhibitor 28% <i>response</i> <sup>217</sup>
GnT-V,β1, 6-N <sup>29, 183, 209, 220</sup>	b 1.6 N-acetyl glucosaminotransferase	Astrocytoma, Adult GBM	N/A
GP-100 <sup>29, 50, 132, 143, 183, 221, 222</sup>	Melanoma Glycoprotein-100	Low expression in adult and pediatric brain tumors	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13Rα2, and AIM-2 peptides 33% <i>immune response</i> <sup>132</sup> DC vaccine pulsed with EphA2, IL-13Rα2, YKL-40, and gp100 (NCT00766753) 58% <i>immune response</i> <sup>143</sup> DC vaccine pulsed with WT-1, HER2, MAGE-A3,

			and MAGE-A1 or gp100 <i>66% cellular immune response</i> <sup>221</sup>
Her-2/Neu <sup>29, 132, 183, 209, 221-223</sup>	Human Epidermal Growth Factor Receptor 2, proto-oncogene Neu, receptor tyrosine-protein kinase erbB-2, CD340	Adult GBM	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2, and AIM-2 peptides <i>33% immune response</i> <sup>132</sup> DC vaccine pulsed with WT-1, HER2, MAGE-A3, and MAGE-A1 or gp100 <i>66% cellular immune response</i> <sup>221</sup> HER-2 CAR (NCT02442297) <i>ongoing</i>
IDH1R132H <sup>224</sup>	Isocitrate Dehydrogenase 1 gene	Mutations present in majority of several types of malignant glioma, most prevalent in secondary GBM	PEPIDH1M: IDH1 peptide vaccine (NCT02193347) <i>ongoing</i> IDH1 peptide vaccine (NOA-16, NCT02454634) <i>ongoing</i>
IL-13R $\alpha$ 2 <sup>50, 132, 143, 183, 207, 210, 225, 226</sup>	Interleukin 13 receptor alpha 2 protein chain	High grade astrocytoma	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2, and AIM-2 peptides <i>33% immune response</i> <sup>132</sup> DC vaccine pulsed with EphA2, IL-13R $\alpha$ 2, YKL-40, and gp100 (NCT00766753) <i>58% immune response</i> <sup>143</sup> EphA2, IL-13R $\alpha$ 2, and survivin peptide vaccine (NCT01130077) <i>62% peptide specific immune response</i> <sup>131</sup> SL-701 peptide vaccine (NCT02078648) <i>ongoing</i>

			IL13R $\alpha$ 2 CAR (NCT02208362) <i>ongoing</i>
Ki-67 <sup>189, 227</sup>	The nuclear cell proliferation-associated antigen of antibody Ki-67	Prognostic marker for low grade glioma	N/A
Ku70/Ku80 <sup>228</sup>	Human Ku heterodimer proteins subunits	Glioblastoma	N/A
LICAM <sup>229, 230</sup>	L1 Cell Adhesion molecule	Glioma stem cells	N/A
Mage-1 <sup>29, 50, 132, 183, 221, 222, 231</sup>	Melanoma Antigen-1	Adult GBM, Neuroblastoma	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2, and AIM-2 peptides <i>33% immune response</i> <sup>132</sup>  DC vaccine pulsed with WT-1, HER2, MAGE-A3, and MAGE-A1 or gp100 <i>66% cellular immune response</i> <sup>221</sup>
Mage-3 <sup>221, 231</sup>	Melanoma Antigen-3	Adult GBM, Neuroblastoma	DC vaccine pulsed with WT-1, HER2, MAGE-A3, and MAGE-A1 or gp100 <i>66% cellular immune response</i> <sup>221</sup>
MART-1 <sup>29, 183</sup>	Melanoma antigen recognized by T-cells	Low expression in adult and pediatric brain tumors	N/A
MRP3 <sup>29, 232</sup>	Multidrug Resistance Protein-3	Adult GBM	Personalized peptide vaccine mix containing MRP3 peptide <i>83% peptide-specific CTL response to at least one peptide</i> <sup>233</sup>
Nestin <sup>197, 198, 215, 231, 234</sup>	Nestin	Glioma stem cells, Glioblastoma, Astrocytoma	N/A
NY-ESO-1 <sup>231, 235</sup>	New York esophageal squamous cell	Neuroblastoma	CDX 1401 vaccine: DEC-205 antibody linked to NY-ESO-1 (NCT00948961) <i>79% NY-ESO-1-specific</i>

	carcinoma 1		<i>antibody response, 56% NY-ESO-1-specific T cell response</i> <sup>235</sup>  CDX-1401 vaccine (NCT00948961) <i>ongoing</i>
Olig2 <sup>215, 236-238</sup>	Oligodendrocyte transcription factor 1	Glioma stem cells, Oligodendroglioma, Oligoastrocytoma	N/A
Prox1 <sup>239</sup>	Prospero homeobox protein 1	High grade glioma (also in immature neurons)	N/A
PTH-rP <sup>29</sup>	Para-Thyroid Hormone-related Protein	Adult GBM	N/A
PSCA <sup>240</sup>	Prostate stem cell antigen, GPI-anchored cell surface protein	WHO grade III-IV gliomas	N/A
Sart-1 <sup>29, 183</sup>	Squamous cell carcinoma Antigen Recognized by T cells-1	Astrocytoma, Adult GBM	N/A
Sart-2 <sup>29</sup>	Squamous cell carcinoma Antigen Recognized by T cells-2	Adult GBM	N/A
Sart-3 <sup>29</sup>	Squamous cell carcinoma Antigen Recognized by T cells-3	Adult GBM	N/A
Sox 2 <sup>241, 242</sup>	SRY (sex determining region Y)-box 2	Glioma stem cells	N/A
Sox 6 <sup>243</sup>	SRY (sex determining region Y)-box 6	Glioma stem cells	N/A
Sox 10 <sup>236</sup>	SRY (sex determining region Y)-box 10	Oligodendroglioma, Oligoastrocytoma	N/A
Sox 11 <sup>29, 209, 244</sup>	SRY (sex determining region Y)-box 11	Adult and pediatric brain tumors	N/A
SSEA <sup>245</sup>	Stage-Specific Embryonic	Glioma stem cells	N/A

	Antigen 1(SSEA-1/LeX)		
Survivin <sup>29, 183, 209, 227</sup>	Survivin	Glioblastoma	EphA2, IL-13R $\alpha$ 2, and survivin peptide vaccine (NCT01130077) <i>62% peptide specific immune response</i> <sup>131</sup>  SL-701 peptide vaccine (NCT02078648) <i>ongoing</i>  SurVaxM survivin peptide vaccine (NCT02455557) <i>ongoing</i>
h-Tert <sup>29, 246</sup>	Telomerase Reverse Transcriptase	Adult GBM	N/A
TRP-1 <sup>29</sup>	Tyrosinase Related Protein-1	Adult GBM	N/A
TRP-2 <sup>29, 50, 132</sup>	Tyrosinase Related Protein-2	Adult and Pediatric brain tumors	DC vaccine pulsed with HER2, TRP-2, gp100, MAGE-1, IL13R $\alpha$ 2, and AIM-2 peptides <i>33% immune response</i> <sup>132</sup>
Tyrosinase <sup>29</sup>	Tyrosinase	Adult GBM	N/A
UPAR <sup>247, 248</sup>	Urokinase-type plasminogen activator receptor, CD87	High grade glioma cells	N/A
WT-1 <sup>221, 249-251</sup>	Wilm's Tumor Antigen – 1, WT2725	Glioblastoma and Medulloblastoma	DC vaccine pulsed with WT-1, HER2, MAGE-A3, and MAGE-A1 or gp100 <i>66% cellular immune response</i> <sup>221</sup>  WT-1 peptide vaccine <i>No increase in WT-1 specific CTLs post-vaccination</i> <sup>249</sup>  WT2725: WT-1 peptide vaccine (NCT01621542) <i>ongoing</i>
YKL-40 <sup>29, 143, 236, 252</sup>	Chitinase-3-like protein 1 (CHI3L1), also known as	Astrocytoma, Oligoastrocytoma, High	DC vaccine pulsed with EphA2, IL-13R $\alpha$ 2, YKL-40,

	YKL-40	grade glioma, Glioma Stem Cells, secreted biomarker present in the serum of glioblastoma patients, Adult and pediatric brain tumors	and gp100 (NCT00766753) <i>58% immune response</i> <sup>143</sup>  Peptide vaccine with EGFRvIII, IL-13R $\alpha$ 2, EphA2, Her2/neu, YKL-40 peptides (NCT02754362) <i>ongoing</i>
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**Table 3: Key factors that impact the efficacy of cancer vaccines for GBM patients**

Strategy	Items
Generation of tumor antigens <sup>143, 144, 221, 253-259</sup>	CMV pp65 RNA, IDH1(R132H), WT1, EGFRvIII, IL13R-a2, H3F3A(K27M), autologous tumor lysate, HSPPC-96, cocktail of peptides
Delivery of tumor antigens <sup>87, 260-263</sup>	systemic injection of antigens, dendritic cells, (viral vector)
Adjuvant <sup>142, 174, 264, 265</sup>	tetanus, KLH, ISA51, imiquimod, polyI:CLC, (anti-PD-1 Ab, anti-CTLA4 Ab, anti-Gr1 MDSC inhibitor, IDO inhibitor)
Stratification of patients <sup>142, 169, 170</sup>	pSTAT signaling, mesenchymal gene expression signature, TCR

GBM, glioblastoma; CMV, cytomegalovirus; pp65, phosphoprotein 65; IDH1, isocitrate dehydrogenase 1; WT1, Wilms tumor 1; EGFR, epidermal growth factor receptor; HSPPC, heat shock protein peptide complex; PD-1, programmed death 1; CTLA4, cytotoxic lymphocyte antigen 4; MDSC, myeloid derived suppressor cell; IDO, indoleamine 2,3-dioxygenase; STAT, signal transducer and activator of transcription; TCR, T cell receptor. Adjuvants in the parenthesis have not been tested in human trials.

#### ARTICLE HIGHLIGHTS BOX

- Molecular features of gliomas such as 1p/19q co-deletion, IDH1 mutation, mutation in TERT gene and loss of ATRX are increasingly used in the diagnosis of tumor samples and are predictive of histological sub-type and prognosis.
- GBM TME is highly immunosuppressive because of the presence of cells such as Tregs, MDSCs and TAMs, secretion of IL-10 and TGF- $\beta$  by GBM cells, differential regulation of HLA

molecules on GBM cells and through the expression of T cell suppressive molecules such as CD80 and PDL1. Thus multipronged strategies are needed to overcome the GBM induced immune suppression effectively.

- Immunotherapeutic strategies targeting GBM can be broadly classified as immunomodulatory approaches, active immunotherapy, passive immunotherapy and adoptive immunotherapy using autologous T cells and CAR T cells.
- Immunomodulatory approaches target immunosuppressive checkpoints such as CTLA-4 and PD-1. A clinical trial evaluating Ipilimumab for recurrent and newly diagnosed GBM is currently recruiting patients (NCT02017717). Another immunomodulatory therapy utilizes gene therapy. The conditionally cytotoxic-immune-stimulatory gene therapy approach developed in our laboratory, has proven to be highly efficacious in several rat and mouse intracranial GBM models and is currently being tested in the clinic (NCT01811992).
- Active immunotherapy aims at stimulating innate and adaptive immune responses against tumor-specific antigens. Several known tumor associated antigens such as HER-2, TRP-2, gp100, MAGE-1, IL-13 $\alpha$ 2, EphA2 and AIM-2, are being targeted in GBM. Neoantigens such as EGFRvIII are increasingly being used to generate tumor specific responses. Mutant IDH1 peptide-derived vaccine PEPIDH1M is being tested in patients with IDH1 positive recurrent grade II glioma (NCT02193347) and the NOA-16 trial is a Phase I trial of IDH1 peptide vaccine for grade III-IV gliomas (NCT02454634).
- Adoptive immunotherapy using autologous T cell transfer and CAR T cells is also being tested. A phase I trial will test the safety and efficacy of using HER2-specific CARs in patients with recurrent GBM (NCT02442297). The Rosenberg group at NCI (NCT01454596) and the University of Pennsylvania/Novartis (NCT02209376) are also currently recruiting participants to test the safety and feasibility of administering T cells expressing anti-EGFRvIII CAR to GBM patients who have had their first recurrence or have residual disease after initial resection.