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Graphical abstract

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Treating ICB-resistant glioma with anti-CD40 and mitotic spindle checkpoint controller BAL101553 (lisavanbulin)

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Conflicts of interest.

PMS, FB and HL own stocks and are employees of Basilea Pharmaceutica International Ltd.
PRW is a consultant for Basilea Pharmaceutica International Ltd.
VG, FIE, EM, VB and PYD declare no potential conflict of interest.
Abstract

Glioblastoma is a highly malignant brain tumor with no curative treatment options, and immune checkpoint blockade has not yet shown major impact. We hypothesized that drugs targeting mitosis might impact the tumor microenvironment and sensitize cancer cells to immunotherapy. We used two glioblastoma mouse models with different immunogenicity profiles, GL261 and SB28, to test the efficacy of antineoplastic and immunotherapy combinations. The spindle assembly checkpoint activator BAL101553 (lisavanbulin), agonistic anti-CD40 antibody, and double immune checkpoint blockade (anti-PD-1 and anti-CTLA-4) were evaluated individually or in combination for treating orthotopic GL261 and SB28 tumors. Genomic and immunological analyses were used to predict and interpret therapy responsiveness. BAL101553 monotherapy increased survival in immune checkpoint blockade resistant SB28 glioblastoma tumors and synergized with anti-CD40 antibody, in a T-cell independent manner. In contrast, the more immunogenic and highly mutated GL261 model responded best to anti-PD-1 and anti-CTLA-4 therapy and more modestly to BAL101553 and anti-CD40 combination. Our results show that BAL101553 is a promising therapeutic agent for glioblastoma and could synergize with innate immune stimulation. Overall, these data strongly support immune profiling of glioblastoma patients and preclinical testing of combination therapies with appropriate models for particular patient groups.
**Introduction**

Glioblastoma (GBM), the most prevalent primary brain tumor, is a particularly heterogeneous and aggressive tumor, with a poor prognosis of less than 2 years of expected survival at diagnosis (1). Current standard of care is comprised of surgery followed by radiotherapy and temozolomide (TMZ), with no improved treatment protocols for over a decade (2). For other cancer indications, immunotherapies have brought unprecedented clinical benefits for patients (3) and there is optimism that similar treatment approaches could be adapted for GBM.

Although anti-tumor immunity can employ diverse effector mechanisms, clinical cancer immunotherapy has become dominated by different immune-checkpoint blockade (ICB) strategies, with PD-1/PD-L1 and CTLA-4 blocking antibodies (Ab) already approved for multiple indications (4). Nevertheless, it is not clear whether it is a realistic objective to extend ICB to a majority of cancer indications, as most patients are still unresponsive, with lack of T cell infiltration (5) or low mutational load (6) being linked to this ICB resistance. For GBM, most tumors exhibit a low mutational load with few infiltrating T cells and they can be described as an immunologically “cold” tumor (7). Mutational load is linked to immunotherapy through neoepitopes that originate from mutated proteins and that are highly immunogenic (8), but need to be presented to T cells by MHC-I, which is downregulated in human GBM (9). Moreover, GBM generates a highly immunosuppressive tumor microenvironment (TME), with high infiltration of potentially pro-tumoral regulatory T cells (Treg) and M0/M2 macrophages (10, 11).

In several pre-clinical studies, “cold” tumors have been sensitized to ICB monotherapy, by combining different treatment strategies such as antineoplastic agents, or by targeting multiple immune checkpoints (12), with some treatment combinations also tested in GBM models (13, 14). However, as we have previously shown (15), the baseline ICB response of
mouse GBM models can vary considerably, likely mirroring very different patient groups. Therefore, in order to investigate combination treatment for GBM in studies that will translate to clinical application, the choice of mouse model is decisive. The GL261 glioma mouse model is highly sensitive to ICB (15, 16), and may recapitulate responses of certain, but rare GBM patients, whereas the SB28 model will better represent the immunotherapy sensitivity of the majority of human GBM (15).

In this study we used both SB28 and GL261 mouse GBM models to explore therapeutic combinations, including ICB, with a focus on the more stringent and challenging SB28 model. In view of the major myeloid cell infiltration but minor T cell infiltration of SB28 tumors (15), we anticipated that both immune compartments would require modulation to achieve therapeutic effect. We hypothesized that drugs inducing mitotic spindle checkpoints would induce genomic instability in tumor cells, as a first step towards sensitizing them to ICB. Hence, we investigated the use of BAL27862 (avenbulin) and its prodrug BAL101553 (lisavanbulin) (17), a novel microtubule disrupting agent (18), whose activity is dependent on activation of the mitotic spindle assembly checkpoint (SAC) (19); which has high blood-brain-barrier (BBB) penetrance (20) and activity in orthotopic GBM models (21). BAL101553 is currently being evaluated in phase I/IIa trials for GBM as monotherapy or in combination with radiotherapy (ClinicalTrials.gov Identifier: NCT03250299 and NCT02490800). This compound showed in vitro and in vivo activity in the two models used, SB28 and GL261. Immunotherapeutic strategies alone, including anti-PD-1 (aPD-1) and anti-CTLA-4 (aCTLA-4) ICB, or agonistic anti-CD40 (aCD40) Ab, acting on macrophages and DCs, were not efficacious in the stringent SB28 model, but a synergistic effect leading to prolonged survival was obtained with a combination of aCD40 and BAL101553. Analysis of SB28 mutational load and the immune infiltrate suggested that this combined therapy might be able to function independently of T cells, which was confirmed by reproducing the therapeutic effect in T and B cell deficient mice.
The same treatment strategies were also tested in the more immunogenic GL261 model, which revealed a favorable outcome when BAL101553 was combined with aCD40, but not with ICB. Overall, these results indicate that, with an optimal combination of treatment modalities, a synergistic effect of selected antineoplastic drugs and immunotherapy agents can be obtained even in a stringent preclinical model of GBM.
Results

**BAL27862 inhibits SB28 glioma cell proliferation in vitro and stimulates HMGB1 release**

BAL27862 has a cytostatic and cytolytic effect on SB28 cells (Supplementary Figure S1), with an IC50 of 5.5nM (Fig. 1A). We also assessed BAL27862 induction of key molecules associated with immunogenic cell death (ICD), namely, extracellular release of ATP and HMGB1, and translocation of calreticulin to the membrane. BAL27862 did not induce extracellular release of ATP, nor calreticulin translocation (Fig. 1B and C). In contrast, the compound modestly increased extracellular release of HMGB1 at all concentrations tested (Fig. 1D). These results indicate that SB28 is sensitive to BAL27862 at in vivo achievable concentrations, and that the resulting dying cells might stimulate recognition by innate immune cells through release of HMGB1.

**BAL101553 prolongs survival of mice bearing orthotopic, temozolomide resistant SB28 glioma, but does not sensitize to ICB**

We assessed the efficacy of TMZ treatment in mice implanted intra-cranially (IC) with SB28 (Fig. 2A), a protocol previously reported to be efficacious in the GL261 model (22). SB28 implanted mice were resistant to TMZ therapy, with no statistically significant increase in survival (Fig. 2B). We then proceeded to in vivo testing with the prodrug BAL101553 to determine whether the in vitro benefits of BAL27862 could be recapitulated in vivo. BAL101553 monotherapy resulted in a statistically significant prolonged survival of implanted mice (Fig. 2C), resulting from delayed SB28 growth assessed by in vivo bioluminescence imaging (Supplementary Figure S2). However, once tumor growth was unequivocal, growth rate was similar in control and BAL101553 treated mice. Histological analysis of brains at an intermediate timepoint (21 days post-implantation) also showed a clear reduction in tumor size
in BAL101553 treated mice compared to controls (Supplementary Figure S3). To further optimize BAL101553 effect, we tested treatment durations of 2 and 4 weeks, or until appearance of terminal symptoms. The latter treatment duration significantly increased survival compared to 2 weeks (42 vs 35 days of median survival (MS), but not with 4 weeks of treatment (Supplementary Figure S4A and B).

We next evaluated the effects of combining BAL101553 treatment with ICB (consisting of a combination of aPD-1 and aCTLA-4) using an established protocol previously shown to be efficacious in the GL261 mouse glioma model, but not in SB28 (15). We confirmed resistance of SB28 implanted mice to ICB alone, as no survival increase was observed. Moreover, combination of BAL101553 with ICB did not reveal any statistically significant impact over BAL101553 treatment alone (MS: 35 days vs 39 days for BAL101553 or BAL101553 and ICB, respectively) (Fig. 2C).

BAL101553 is an activator of the SAC, inducing cell cycle arrest and subsequent death, or aberrant chromosome segregation leading to genomic instability (23). This in turn could induce mutations and promote neoeptope generation, thereby potentiating the ICB effect. We hypothesized that late combination with ICB, when BAL101553 had already impacted tumor cells for an extended time period, might be most efficacious. Moreover, we reasoned that even low doses of BAL101553, not leading to immediate cell death, could also efficiently drive genomic instability. We therefore tested late combination of ICB and a lower dose (15 mg/kg) of BAL101553. However, late use of ICB, either alone or after extended BAL101553 treatment did not reveal any ICB/BAL101553 combination effect (Supplementary Figure S4C).

Regarding low-dose treatment with BAL101553 at 15mg/kg, this was as potent as 25mg/kg to prolong survival: 40 and 42 days of MS for 15mg/kg and 25mg/kg, respectively, compared to 29 days of MS for controls. However, the lower dose of BAL101553 still did not induce ICB responsiveness (Supplementary Figure S4D).
SB28 cells acquire an IFNγ–response signature in vivo

RNA sequencing was used to assess transcriptional changes in flow cytometry sorted SB28 cells resulting from 21 days or 33 days of in vivo growth (mice treated with vehicle control or BAL101553, respectively). There was a prominent upregulation of expression of IFNγ response genes in ex vivo samples compared to their in vitro counterparts (Fig. 3A). Expression of B2m, H2.D1 and H2.K1, coding for MHC-I chains, were significantly upregulated. Other IFNγ-regulated genes such as Fn1, Lgals3 and Stat1 were also expressed at a significantly higher level by ex vivo samples. Interestingly, Pdl1, which is included in the IFNγ signature, was significantly upregulated, as well as other genes encoding (potentially) immunoregulatory molecules such as CD80, CD155, Tgfβ1 and CD276 (B7H3), but which are not part of the IFNγ signature (Figure 3B). In order to validate the presence of IFNγ cytokine that could induce this pattern of gene expression by the tumor cells, we tested whole tumor lysates with an IFNγ ELISA (data not shown); this showed a trend for more IFNγ in tumor bearing mice compared to healthy brains, presumably derived from infiltrating T cells and NK cells. In contrast to the in vitro and ex vivo differences in gene expression by SB28 cells from non-treated mice, no significant differences were observed between ex vivo controls and SB28 cells sorted from BAL101553 treated mice, neither for the IFNγ signature, nor for immunoregulatory molecule expression (Supplementary Figure S5A and B).

BAL101553 synergistically combines with aCD40 to increase survival

Because SB28 is an immunologically “cold” tumor (15), and because BAL101553 therapy cannot potentiate an ICB effect in two combination treatment protocols, we hypothesized that immunogenicity of SB28 might be augmented by immune activation in addition to ICB blockade. We tested an agonistic aCD40 Ab, reasoning that this may promote macrophage and
dendritic cell (DC) activation, which in turn would promote T-cell dependent anti-tumor immunity. We first tested a late combination with ICB, in order to have tumor impacted by BAL101553 treatment, and aCD40 used concomitantly with ICB (Supplementary Figure S6A). Adding agonistic aCD40 alone did not prolong survival (Supplementary Figure S6B); all survival-extending treatments required BAL101553, either alone or in combination with aCD40 or with ICB. The modest, but non-significant trend for prolonged survival with the BAL101553 and aCD40 combination encouraged us to try to enhance the effect, and to explore potential mechanisms. Because we had determined that prolonged exposure to BAL101553 did not significantly increase mutational load (Supplementary Figure S7), there was less rationale for late combination of immunotherapy. We hypothesized that combining aCD40 from day 7 post-implantation (as in Fig. 4A) might enhance its combinatorial effect. Indeed, there was a modest but significant increase of survival when BAL101553 was combined with aCD40, compared to BAL101553 monotherapy (MS of 42 days and 49 days in BAL101553 and BAL101553 plus aCD40 groups, respectively). This combination treatment resulted in an 81% increase in survival compared to control mice (Fig. 4B). Notably, aCD40 monotherapy was inefficacious, with combination therapy needed to reveal a synergistic treatment effect.

**Efficacy of BAL101553 and aCD40 combination therapy is T-cell independent, and impacts on BMDM tumor infiltration**

To identify immune subsets modulated by the partially efficacious BAL101553 plus aCD40 combination therapy, we analyzed BILs at different time points. The immune infiltrate was significantly impacted by the different treatments, in particular with aCD40 monotherapy (Supplementary Figure S8), with putative pro-tumoral (red) or anti-tumoral (green) immune subsets indicated for the different treatment protocols. Complete analyses of mice sacrificed at appearance of terminal symptoms showed a significant increase in infiltrating T cells, with a
phenotype compatible with less exhausted (PD-1⁺, KLRG1⁻) but more memory (PD-1⁻, KLRG1⁺) CD8 T cells, and a higher CD8/CD4 and CD8/Treg ratio in the aCD40 treated group (Supplementary Figure S8G-I) (24). BAL101553 monotherapy also impacted on BILs, with higher MHC-II expression by DCs, and more natural killer (NK) cell infiltration at d33 after tumor implantation (Supplementary Figure S8J and S8K), but this effect was lost at the time of terminal symptoms. Notably, upon appearance of terminal symptoms, mice treated with BAL101553, as monotherapy or in combination with aCD40, showed lower macrophage expression of PD-L1 compared to controls or aCD40 treated mice (Supplementary Figure S8O).

Overall, considering flow cytometry data and immunohistology (data not shown), no single innate or adaptive immune subset could be directly correlated with the synergistic effect of BAL101553 and aCD40. To gain insight into roles of innate versus adaptive immune cells, we repeated the same treatment regimens with tumor bearing B6.129S7-Rag1tm1Mom/J (RAG1 KO) mice, lacking T and B lymphocytes. The survival advantage offered by aCD40 monotherapy and the synergistic effect of BAL101553 and aCD40 seen in immunocompetent mice were fully recapitulated in these T and B cell immunodeficient mice (Fig. 4C). These results unequivocally exclude T cells (or B cells) as being involved in the therapeutic effect of this antineoplastic and immunomodulatory combination treatment, and instead implicate innate immune cells, present in both mouse strains.

Considering the T cell independence for the therapeutic effect of BAL101553 and aCD40 combination, we conducted more detailed analysis of myeloid cells infiltration. In Figure 5, we see significantly less BMDM tumor infiltration compared to microglia cells at day 23 after tumor implantation. Moreover, PD-L1 expression of BMDM was significantly enhanced after combination therapy when compared with single modality treatments. DC subsets were also characterized, with higher MHCII expression (Supplementary Figure S8J),
but lower cDC2 (CD45\(^+\)CD11c\(^+\)CD8\(^-\)) infiltration in mice treated with BAL101553 (Supplementary Figure S9).

**Combination of BAL101553 and aCD40 is also efficacious in GL261 implanted mice**

In order to assess the potential of therapeutic combinations of ICB and aCD40 with BAL101553 in more highly infiltrated and mutated GBM, we also tested treatment combinations in GL261-bearing mice (15) (Fig. 6A). GL261 cells were less sensitive than SB28 to BAL27862 in vitro, with an IC50 of 13.4nM (Supplementary Figure S10). Consistent with this, BAL101553 monotherapy, using the same regime that was efficacious in SB28 bearing mice, did not impact survival in the GL261 model (Fig. 6B). However, when BAL101553 was combined with aCD40 (inefficacious as monotherapy), there was a significant survival benefit, although all mice eventually succumbed to tumor growth. This contrasts to the efficacy of ICB therapy in this model, which, as expected in this highly mutated tumor (15, 25), led to long term survival in 7/10 mice. Surprisingly, combination of BAL101553 and ICB had a significantly lower effect than ICB alone, with only 1/10 mice surviving at the end of the follow-up (100 days post tumor implantation) (Fig. 6B).
Discussion

There is a major unmet clinical need to develop new treatments for GBM, but immunotherapy is proving challenging to use effectively. Indeed, a phase III clinical trial (26) showed no benefits from aPD-1 therapy compared to bevacizumab, although use of nivolumab in a neo-adjuvant setting was more encouraging (27, 28). Nevertheless, more widespread application of immunotherapies for GBM will require stringent pre-clinical testing using models that recapitulate both ICB-responsive tumors and ICB-resistant tumors, exemplified by the GL261 and SB28 models previously described (15) and further investigated in the present study. Our results show that even in the poorly mutated and immunogenic SB28 GBM model, there is still evidence for immune activation, with IFNγ measured in the tumor (data not shown) and an IFNγ response gene signature detected ex vivo. Moreover, PD-L1 was upregulated in SB28 tumors after in vivo growth, validating the rational to use an ICB strategy. However, use of aPD-1 and aCTLA-4 ICB in the context of SB28 treatment never improved survival in our experiments, indicating that further treatment combinations need to be considered. Combination therapies have been successfully used to treat an ICB resistant mouse pancreatic cancer model, using radiation, antineoplastic agents and immunomodulators (12), or with a hypoxia-activated prodrug together with double ICB in transgenic prostate models (29). Even the highly resistant SB28 GBM model showed some responsiveness to a triple therapy comprised of TMZ, a Na/H exchanger blocker and aPD-1, although there were no long-term survivors (30). The number of possible treatment protocols when two, three, or more compounds or modalities are combined will rapidly exceed what is testable clinically, and so maximum insight must be gained from preclinical investigation (31). Moreover, using more than one model is likely to be helpful in order to recapitulate subgroups of patients.
In human GBM, patients with a hypomethylated MGMT do not benefit from the addition of TMZ to radiotherapy (32). Few alternative chemotherapy agents efficiently reach the tumor site in the brain, hence the interest in BAL101553 which efficiently enters the brain of treated rodents (20), is active in human GBM models (21, 33), and is currently undergoing clinical evaluation in GBM patients (ClinicalTrials Identifier: NCT03250299 and NCT02490800). In our experiments, SB28 GBM was also insensitive to TMZ, but responsive to BAL101553 treatment, in vitro and in vivo, with significant survival increase, but no long-term survivors. The initial slower growth rate of SB28 tumors in BAL101553 treated mice (as judged by bioluminescence) would be compatible with a cytostatic action of the compound, which we did observe in vitro, in addition to\$ cytotoxicity. However, the subsequent growth rate was similar in treated and untreated mice, which would be compatible either with an acquired resistance mechanism and outgrowth of resistant cells, or with decreased drug availability to some tumor cells after a certain volume increase. Antineoplastic agents can potentially synergize with immunotherapy if it stimulates innate immunity through ICD (34), or adaptive immunity through enhanced neo-epitope expression. BAL101553 did not strongly induce ICD in SB28 cells, although there was a modest increase in the release of HMGB1, which has been reported to bind to TLR2 and TLR4 and to enhance GBM-infiltration by DCs (35). Consistent with this, we observed higher activation of brain-infiltrating DCs in BAL101553 treated SB28-bearing mice. Some antineoplastic drugs can induce mutations even at sub-cytotoxic doses, leading to neoantigen expression, which may augment tumor immunogenicity. We tested multiple treatment protocols to optimize SB28 exposure to BAL101553, in an attempt to induce mutations and thereby favor ICB responsiveness. However, the time period over which the compound can act on tumor cells in a mouse model is shorter than would occur in human patients, consequently, we observed no significant increase in mutational load after treatment, although the number of predicted neoepitopes was
slightly higher (Figure S7D). Nevertheless, these approaches did not sensitize to ICB. In human GBM, some cases of hypermutated GBM are described, particularly at recurrence after TMZ treatment (36), but their sensitivity to ICB seems limited (37). In contrast, recent encouraging clinical responses were observed after neo-adjuvant ICB in recurrent GBM patients (27, 38) that may reflect higher mutational load after prior TMZ treatment, although this was not reported.

Although ICB is currently championed as the major breakthrough in cancer immunotherapy, there is a considerable fundamental, pre-clinical, and clinical experience in immune stimulation, such as by vaccination, cytokines, or the use of agonistic antibodies (39). Clinical success of such approaches as monotherapies has been limited, but now with so many validated therapeutics available for ICB and for immunostimulation, combining the two approaches for the most refractory cancers is a compelling way forward (40). Among the possibilities, agonistic aCD40 is particularly interesting because it can work at many levels. For example, by activating DCs to more efficiently stimulate T cells, by triggering macrophages to exhibit anti-tumor functions, or even by directly interacting with certain CD40 expressing tumor cells, as proposed for certain human gliomas (41, 42), although this would not be the case for the CD40-negative mouse models used here (15). Use of aCD40 monotherapy for the immunologically cold, SB28 GBM was not surprisingly ineffectivaceous, and indeed was previously reported (43). Nevertheless, aCD40 did favorably modulate infiltration of some immune cell subsets, included increased T-cell infiltration. More interestingly, combining aCD40 with BAL101553 synergistically improved survival, but further improvement was not attained by adding ICB. It is possible that neoantigen expression was still insufficient to be targeted by anti-tumor T cells, as proposed in pancreatic cancer, a malignancy with similarities to GBM, such as resistance to ICB and low mutational load (44). Finally, since aCD40 and BAL101553 treatment of SB28 was equally efficacious in T and B cell deficient mice, this
excludes a role of adaptive immunity, suggesting that SB28 GBM progresses without T-cell mediated immunoediting, and is intrinsically resistant to killing by endogenous T cells. Such tumors might be better targeted by transfer of engineered T cells if suitable antigens could be targeted (45).

Despite the lack of involvement of T cells in the therapeutic response of SB28 to aCD40 and BAL101553, the fact that a robust survival increase was observed highlights the interest of exploiting innate immunity in antineoplastic/immunotherapeutic combinations for poorly antigenic/immunogenic tumors. Myeloid cells, as the major CD40-expressing immune cell in the GBM microenvironment (in humans and also in the SB28 and GL261 mouse models), merit further investigation as a target of immunomodulation. Their origin and phenotype can significantly impact on their function, with M0 and M2 polarized macrophages being described in GBM (11, 46), and a CD73<sup>hi</sup> macrophage signature being associated with poor prognosis (31). Our combination therapy decreased BMDM infiltration compared to microglia, and impacted on PD-L1 expression, suggesting a pro-tumoral role of BMDM and that a reduction in their infiltration favors survival. Moreover, in the GL261 model, PD-L1<sup>+</sup> macrophages were proposed to be responsible for ICB resistance in the proportion of mice unresponsive to treatment (47). Inhibition of CSF1R has been tested to modulate GBM-associated macrophages, but resistance or clinical unresponsiveness was reported (11, 48). Combination therapies will need to be considered, which might also reinvigorate anti-tumor T cells (if present), as shown for a combination of aCD40 and CSF1R inhibitors (49). A further innate immune cell that should be considered is the NK cell, which we observed to be transiently augmented after BAL101553 treatment. The low or absent MHC expression by SB28 (15) would favor NK interaction with GBM cells, although this situation would likely be reversed after NK-secretion of IFNγ, supported by our observation of the IFNγ response signature we detected ex vivo from SB28 bearing mice. As aCD40 and BAL101533 therapy showed some
impact on DCs, this could also favor DC-NK crosstalk (50) in the SB28 model in which the role of T cells appeared to be minimal. The therapeutic interest of NK cells is considerable, particularly in view of their putative roles as effector cells able to kill human stem-like GBM initiating cells (51).

Interestingly, the combination of BAL101553 and aCD40 was also efficacious in the well infiltrated GL261 model, even though these agents were ineffective as monotherapy. However, in view of the characteristics of the GL261 tumor cells, particularly its high mutational load, high MHC-I expression (15), and an intense immune infiltrate, it probably only models a small number of human GBM cases. Unsurprisingly, our results from this study, and those from several other groups, suggest that successful treatments for the GL261 model will be T-cell mediated (13, 52). Notably, the treatment combination of ICB together with BAL101553 was less efficacious than ICB alone, which might represent supraoptimal modulation of the tumor microenvironment, or some cytotoxic/cytostatic action of BAL101553 on T cells during their activation, which we observed in vitro using BAL27862 (data not shown). This situation is unlikely to be encountered in clinical immunotherapy for GBM, particularly if the ICB approach does not include CTLA-4 antagonism. In conclusion, we have identified novel treatment combinations for GBM models that exhibit different responsiveness to the mitotic spindle checkpoint controller BAL101553 or immunomodulators (ICB and aCD40). Poorly immunogenic SB28 GBM, with a low mutational load, was modestly responsive to BAL101553 as monotherapy, and survival was further enhanced by addition of aCD40 stimulation, a treatment that functioned independently of T cells but impacted on BMDM. The more immunogenic and highly mutated GL261 responded to ICB alone (aPD-1 and aCTLA4), but the only therapeutically advantageous combination therapy was BAL101553 and agonistic aCD40. Our results highlight that the immune characteristics of the tumor may override the cancer cell of origin in predicting therapy response treatment combinations, a
factor that should be considered for selecting patients for testing future therapy combinations in GBM.
Methods

Cell lines
SB28 and GL261 murine cell lines (kindly provided by H. Okada, UCSF, San Francisco, USA) were tested as mycoplasma-negative and were cultured as previously described (15). SB28 expresses GFP and luciferase, used for cell sorting, immunofluorescence, and in vivo bioluminescence monitoring.

In vitro tests on SB28 cells
SB28 cells were seeded in 96 well plates for 24h, then further incubated for 48h with the indicated concentrations of BAL27862 (provided by Basilea Pharmaceutica International Ltd, Basel, Switzerland) of 0-60 nM, or the corresponding solvent concentration (DMSO). The median GI50 of BAL27862 across a panel of tumor cell-lines is 12 nM; a concentration readily achievable in mouse plasma based on the Cave (AUC0-24h/24) following a dose of 20 mg/kg, per os (Basilea data on-file). CellTiter-Glo Luminescent Cell Viability/Proliferation kit (Promega) was used to assess viable cell number based on total ATP content. Culture supernatants were assessed for extracellular ATP and HMGB1 release by an ENLITEN ATP Assay (Promega) and an HMGB1 ELISA (Tecan), respectively. Cell surface calreticulin Ab was assessed by flow cytometry on detached cells using ab2907 Ab (Abcam).

Brain infiltrating lymphocytes (BILs) isolation and flow cytometry analysis
Mice were sacrificed, perfused trans-cardially with Ringer’s solution and BILs were partially purified as described previously (53). Cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen), blocked for Fc receptor binding with 2.4G2, then surface stained with the following Ab: CD45 (30-F11), CD11b (M1/70), CD49d (R1-2), CD62L (Mel14),
Ly6G (1A8), Ly6C (HK1.4), CD103 (M290), CD8 (53–6.7), CD4 (GK1.5), PD-1 (29F.1A12), PD-L1 (MIH5), NK1.1 (PK136), MHC-II (I-A/I-E, M5/114.15.2), CD80 (16-10A1), CD86 (GL-1), CD11c (N418), CD3 (145-2C11) and KLRG1 (2F1/KLRG1). For intracellular staining, we used Cytofix/Cytoperm kit (BD Biosciences) and stained for FoxP3 (FJK-16s). All Ab were purchased from Biolegend or BD Biosciences. Stained cells were analyzed on a Gallios flow cytometer (Beckman Coulter) and data analyzed used Kaluza software (Beckman Coulter).

**Proliferation and apoptosis assay on SB28 cells**

SB28 cells were incubated with CellTrace™ Violet (Invitrogen) and seeded in 12 well plates for 24h. They were then further incubated for 48h with the indicated concentrations of BAL27862 (provided by Basilea Pharmaceutica International Ltd, Basel, Switzerland), or the corresponding solvent concentration (DMSO). Cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) and Annexin V PE-conjugated (Immunotools). Stained cells were analyzed on a Gallios flow cytometer (Beckman Coulter) and data analyzed used Kaluza software (Beckman Coulter).

**Mouse implantation, bioluminescence and in vivo treatment**

Tumor cells were implanted orthotopically as previously described (15), using 6-8-week-old C57BL/6J mice (Charles River) or B6.129S7-Rag1<sup>tm1Mom</sup>/J mice (The Jackson Laboratory). Mice were monitored daily for weight loss or symptom appearance and euthanized according to veterinary authorized endpoints. For bioluminescence monitoring, mice were injected intraperitoneally (IP) with 3mg of Luciferin (Gold Biotechnology) 5 minutes prior to image acquisition on an IVIS Spectrum system (Xenogen, PerkinElmer) with 3 images collected at 5-minute intervals. Only the highest signal was used for quantification. Therapeutic Ab were all purchased from BioXcell: anti-PD-1 (RMP1-14), anti-CTLA-4 (9D9), anti-CD40 (FGK4.5),
rat IgG2a isotype control (2A3), and mouse IgG2b isotype control (MPC-11) were injected IP as specified. The prodrug BAL101553 was provided by Basilea Pharmaceutica International Ltd., and was administered by oral gavage at the indicated concentrations. TMZ was purchased from Sigma-Aldrich and injected IP at 50mg/kg dose.

Ex vivo cell sorting

At day 21 or 35 after tumor implantation, for controls or BAL101553 treated mice respectively to match for tumor volume, mice were sacrificed, and brains dissected. Tumor cells were obtained using a Brain Dissociation kit (Miltenyi Biotec), staining with anti-CD45 (30-F11) and anti-CD11b (M1/70) Ab from Biolegend, then sorting on a FACS Aria II (Becton Dickinson), gating on CD45+/CD11b+/GFP cells. We collected a total of 300,000 cells per sample.

Sequencing data and analysis

We extracted DNA and RNA from the sorted samples using an AllPrep DNA/RNA Mini Kit (Qiagen). Agilent protocol with 100 pair-end reads was used for whole exome sequencing (WES). To analyze the data, we used the following programs: MuTect or Haplotype Caller for variant calling and SNPeff for variant annotation. We report single nucleotide variant (SNV) and frameshift mutations for each sample. NetMHCPan 4.0 or NetMHCIIIPan 4.0 and IEDB v2.18 were used to predict neoepitope binding to MHC-I and II. TruSeq stranded RNA protocol on Illumina was used for RNA sequencing, and the 50bp reads were mapped with TopHat v2.3.13. PicardTools v1.80 was used for quality control and summarization. HTSeq and Rsubread featurCounts obtained the counts. Differential expression analysis after normalization was performed by R/Bioconductor package edgeR v3.4.3. For interferon gamma (IFNγ) signature and differential expression of immunoregulatory molecules, we performed waterfall
analysis using R. We sequenced 2 ex vivo samples for each control mice or BAL101553 group, and report the results as median of both samples, or a combination of all genes expressed in the 2 samples. RNA sequence data is available on GEO, accession number: GSE127075.

**Histology and immunofluorescence**

Brains were collected after trans-cardiac perfusion, then transferred to a solution of 30% sucrose overnight. Tissues were then imbedded in Tissue-Tek OCT (Sakura), frozen with liquid nitrogen and stored at -80°C. We used a Leica cryostat for sectioning. Sections were fixed with 4% PFA and blocked with a solution of 2.5% Goat Serum and 5% BSA (Sigma-Aldrich). The following primary Ab were used: rabbit anti-GFP (Proteintech, 50430-2-AP), rat anti-CD31 (Biolegend, MEC13.3), rat anti-F4/80 (BD Biosciences, T45-2342), rat anti-Ki67 (BD Biosciences, B56), and rat anti NK1.1 (Biolegend, PK136). For the secondary Ab, we used: goat anti-Rabbit AF488 (Abcam) and mouse anti-rat eFluor 660 (eBioscience, r2a-21B2). Image acquisition was performed on a Zeiss Axio Imager Z1, Axio Imager.Z2 Basis LSM 800, or a Zeiss Axioscan.Z1 microscope with either x20 or x40 objectives and processed using Zeiss Zen pro software.

**Study approval**

All animal experimental studies were reviewed and approved by institutional and cantonal veterinary authorities in accordance with Swiss Federal law.
**Author contributions.**

Conceived and designed the experiments: VG PRW

Performed the experiments: VG FIE VB

Analyzed the data: VG FIE EM VB PYD PMS FB HL PRW

Wrote the paper: VG FIE EM VB PYD PMS FB HL PRW

**Acknowledgments.**

We thank Prof. Bernhard Wehrle-Haller and Prof. Doron Merkler for their insight, and the core facilities of the University of Geneva for their advice and assistance, especially the iGE3 platform (Mylène Docquier, Didier Chollet and Natacha Civic) for the genomic work, the flow cytometry facility (Cécile Gameiro) and the animal housing facility. We also thank all the members of the Walker and Dietrich groups for helpful comments and discussions, and Sylvie Chliate for assistance with animal experimentation.
References


**Figures**

**Figure 1** – BAL27862 impacts on number of viable SB28 cells in vitro but is a modest immunogenic cell death inducer. (A) Number of viable SB28 cells based on total ATP content by luminescent (in relative light unit) cell viability/proliferation assay; SB28 sensitivity to BAL27862 (BAL) corresponds to an IC50 of 5.466 nM. (B, C, D) Evaluation of BAL27862 induction of key immunogenic cell death (ICD) features: (B) extracellular ATP release; (C) membrane translocation of Calreticulin (calculated as mean fluorescence intensity (MFI) ratio); (D) HMGB1 release. Statistics: Tukey’s test, **:** p<0.0001. Box and whiskers plot with the bounds of the box representing lower and upper quartiles, the line within the box showing the median, and the whiskers showing minimum and maximum values. All results include 3 biological replicates.
Figure 2 – BAL101553 but not temozolomide significantly improves survival of SB28 implanted mice. (A) Treatment schedule of mice intra-cranially implanted with SB28 at day 0. (B) Symptom-free survival curve of mice treated with temozolomide (TMZ) or vehicle control (Ctrl). (C) Symptom-free survival curve of mice treated with anti-PD-1 and anti-CTLA-4 (ICB), BAL101553 (BAL) or a combination of both treatments (ICB+BAL). Treatments were injected intra-peritoneally except for BAL101553 which was administered by oral gavage. Median survival (MS) is displayed in days for survival curves. Statistics: Log-rank (Mantel-Cox): non-significant (ns): p> 0.05; **: p<0.01; ***: p<0.001. n= 10 mice per group.
Figure 3 – Interferon gamma signature and immunomodulatory molecule gene expression by SB28 glioma cells after in vivo growth. (A, B) Gene expression level of genes comprising (A) an interferon-γ (IFNγ) signature and (B) key immunomodulatory molecules, based on RNA sequencing between in vitro and ex vivo SB28 cells, respectively. 2 biological replicates are analyzed for each group.
Figure 4 – Early anti-CD40 combination with BAL101553 therapy offers a significant survival increase in SB28 implanted T/B cell-deficient mice. (A) Treatment schedule of mice intra-cranially implanted with SB28 at day 0. (B, C) Symptom-free survival after treatment with anti-CD40 (aCD40), BAL101553 (BAL) or a combination of both (BAL+aCD40) of (B) wild-type, immunocompetent C57BL/6 mice or (C) immunodeficient B6.129S7-Rag1<sup>tm1Mom</sup>/J (RAG1 KO) mice. Median survival (MS) is indicated in days and the percentage increase (% Incr.) in MS is calculated relative to Ctrl group. Statistics: Log-rank (Mantel-Cox): non-significant (ns) p> 0.05; *: p<0.05; **: p<0.01; ****: p<0.0001. n=8-10 mice per group.
Figure 5 – BAL101553 treated tumors are less infiltrated by BMDM. (A, B) Proportion of brain infiltrating myeloid cells, and their corresponding PD-L1 expression (C, D) from mice intra-cranially implanted with SB28 and treated with vehicle control (Ctrl), anti-CD40 (aCD40), BAL101553 (BAL) or a combination of BAL and aCD40 were collected at d23 (A, C) or at time of terminal symptoms (B, D). Statistics: Tukey’s test, *: p<0.05; ***: p<0.001; ****: p<0.0001. Error bars indicate SD. BMDM cells are defined as (CD11b+CD49d+), MicroGlia as (CD11b−CD49d−). All cells are gated on CD45+ cells after exclusion of doublets and dead cells. MFI: mean fluorescence intensity. Box and whiskers plot with the bounds of the box representing lower and upper quartiles, the line within the box showing the median, and the whiskers showing minimum and maximum values. n=3-4 mice per group.
Figure 6 – Combination of BAL101553 and anti-CD40 significantly improves survival of GL261 implanted mice. (A) Treatment schedule of mice intra-cranially implanted with GL261 at day 0. (B) Symptom-free survival of mice treated with vehicle control (Ctrl), anti-CD40 (aCD40), BAL101553 (BAL), immune checkpoint blockade (ICB), a combination of BAL+ICB, a combination of BAL+aCD40, a combination of ICB+aCD40 and a combination of BAL+ICB+aCD40. Median survival (MS) is displayed in days. Statistics: Log-rank (Mantel-Cox): non-significant (ns) p> 0.05; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.001. n=10 mice per group.
**Graphical Abstract**

**In Brief**

Poorly immunogenic glioblastoma mouse model SB28 is resistant to a combination of immune checkpoint blockade with anti-PD-1 and anti-CTLA-4, but sensitive to a combination of the tumor checkpoint controller BAL101553 and agonistic anti-CD40 stimulation in a T cell independent manner.